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THE CONSTRUCTION OF A MAGNETIC ELECTRON MICROSCOPE OF HIGH RESOLVING POWER[1]

By Albert Prebus[2] and James Hillier[3]

Abstract

A brief historical review of the development of the electron supermicroscope is given. This is followed by a theoretical discussion of the resolving power attainable with the instrument and a summary of the important results which have been obtained by other workers. A description is given of the supermicroscope which has been recently constructed in Toronto. A number of photographs taken with the instrument demonstrate its high resolving power and its applicability as a tool in the sciences.

Introduction

(a) Historical

Modern atomic physics has shown that with a particle of mass *m* travelling at a velocity *v* there is associated a probability function which ascribes characteristics of a wave-train to the moving particle. The wave-length associated with an electron which has been accelerated by a potential difference of *E* volts is given by the de Broglie expression

$$\lambda = \frac{h}{mv} = \sqrt{\frac{150}{E}} \times 10^{-8} \text{ cm.}$$

where *h* is Planck's constant. As the resolving power of present day microscopes is limited by the wave-length of the radiation used to illuminate the specimen under observation, the possibility of utilizing a high velocity electron beam for microscopic observation suggested itself. As shown by H. Busch (12) and C. J. Davisson and C. J. Calbick (14, 15), any axially symmetric magnetic or electrostatic field can be used for focusing an electron beam. We consequently speak of magnetic or electrostatic electron lenses. It was realized quite early that the simple magnetic lens consisting of the magnetic field of a solenoid was the most promising for the attainment of high resolving power and correspondingly high magnifications in the examination of microscopic specimens which do not themselves emit electrons.

Beginning their work in 1931, M. Knoll and E. Ruska (17, 18) devised the first magnetic electron microscope which consisted of two lenses, an objective and a projector. This work culminated in the construction of a two stage microscope by E. Ruska (34, 35) in 1934, the resolving power of which definitely surpassed that of a light microscope. Except for the new "Rastermikroskop" of von Ardenne (2), the above-mentioned instrument is the prototype of all the microscopes which have since been constructed for a similar purpose. A beam of fast electrons (10 to 100 kv.) is incident normally upon an object which is in the form of a thin film. The transmitted beam is focused by means of a magnetic objective lens to form a real image of the object upon a fluorescent screen. Any part of this image may be remagnified by means of a magnetic projection lens to form a final highly magnified image upon a fluorescent screen or photographic plate. Focal lengths of the order of 5 mm. are attainable in each of the lenses. With an image distance of 50 cm. in each stage, a total magnification of 10,000 and greater is possible. Contemporary with this work of Ruska's, a similar microscope was constructed by L. Marton (26–32) in Belgium; the latter made the first attempt to examine biological specimens with such an instrument. A third microscope of this kind was constructed by L. C. Martin, R. V. Whelpton, and D. H. Parnum (24) in England. Owing to technical difficulties encountered, which have been described by L. C. Martin (23), the results obtained with this instrument have been seriously limited. More recently, the Siemens and Halske Allgemeine Gesellschaft, with the assistance of B. von Borries and E. Ruska (8), have constructed a new instrument based on Ruska's design of 1934. This instrument is especially designed for speed and convenience of manipulation and it is capable of a magnification of 56,000 diameters.

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[1] *Manuscript received January 19, 1939.*
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(b) Resolving Limit of a Magnetic Lens

It is of interest to review briefly the main factors which determine the limit of the resolving power, or more concisely, the resolving limit of a magnetic microscope objective lens. The term "resolving limit" may be defined on the basis of one of the factors to be considered, namely diffraction. Of course the actual resolving power of a magnetic lens is limited essentially by other factors to be considered later.

Two neighbouring points in an object which are either self luminous or which scatter incoherently the radiation incident on them from an extraneous source give rise to two independent diffraction patterns of the lens aperture in the plane of the image. The resolving limit R of the lens is defined as the distance between two such neighbouring points when the central maximum of one of their diffraction patterns falls upon the first minimum of the other. The numerical value of R is obtained from the well known formula of Abbé for a light lens with a circular aperture

$$R = \frac{1 \cdot 22\lambda}{2\mu \sin \alpha} \tag{1}$$

where λ is the wave-length of the radiation used;

μ is the refractive index of the medium in which the object is immersed;

and α is the semi-angle of the cone subtended by the aperture of the lens at the object.

In the case of the magnetic lens, the object is situated in a field-free region, so that μ may be set equal to unity; sin α may be replaced by $\frac{D}{2f}$, where D is the aperture diameter of the lens and f is its focal length. This results in the approximate formula

$$R = \frac{1\cdot 22\lambda}{2\cdot\frac{D}{2f}} \tag{2}$$

In the above formula, it is to be observed that by increasing D the value of R can be made as small as desired.

The first factors which prohibit an unlimited increase in D are the third order lens errors. These have been studied by a number of investigators whose results are summarized by Busch and Brueche (13, p. 24 *et seq.*). Of the eight third order errors classified, spherical aberration is of the greatest importance since it affects the quality of the centre of an image as well as its eccentric regions. On the basis of Ruska's experimental work, von Ardenne (1) set up an empirical formula for the transverse spherical aberration which contains the results for one particular set of lens pole pieces of Ruska's microscope objective. This formula is

$$R' = \frac{D}{2}\,.\,K\,.\left(\frac{D}{D_p}\right)^2 \tag{3}$$

where R' is the radius of the circle of least confusion in the image plane divided by the magnification;

K is an empirical factor with the value 0.7;

D is the diameter of the aperture of the magnetic lens which is given approximately by the diameter of the circular opening in the diaphragm which is placed in the gap midway between the pole pieces P (see Fig. 1);

and D_p is the diameter of the narrowest part of the channel which is drilled through the pole pieces and through which the electrons pass.

In this relation, R' increases with D in contrast to the diffraction formula in which R decreases with increasing D.

On the assumptions *(a)* that when the diffraction pattern of a point is very small compared to the transverse spherical aberration, two points are resolved when the distance between them is equal to or greater than R', and *(b)* that when the radius of the circle of least confusion is equal to the radius of the first minimum of the diffraction pattern, two points are resolved for the same values of the radii as if either spherical aberration or diffraction alone were present, Equations (2) and (3) may be solved for $R'(= R)$ and D. With the numerical values

$f = 2.8$ mm.
$E = 75$ kv,

which are attainable with the new microscope of the Siemens and Halske Allgemeine Gesellschaft (8), and a value of D_p assumed by the authors

$$D_p = 3 \text{ mm.}$$

substituted into the equations, the values

$$R = 11 \times 10^{-8} \text{ cm.}$$
$$D = 1.4 \times 10^{-2} \text{ mm.}$$

are obtained. This resolving limit of 11 Å is two orders of magnitude greater than the resolving limit of a highly corrected oil immersion light microscope objective (4, p. 74). To realize this resolving power in practice, however, there are a number of other sources of error to be overcome.

The first of these is the analogue of chromatic aberration. In the first approximation, the focal length of a magnetic lens varies directly as the accelerating potential E of the electron beam. If the beam contains electrons which are accelerated within the range E to $E + \Delta E$, which may be due either to a constant velocity distribution or to a fluctuation in the accelerating potential, then a point image on the optic axis of the lens becomes a disc the radius d of which is given by a formula of von Ardenne (1)

$$d = \frac{D}{2} \cdot \frac{\Delta E}{E}$$

Hence, to attain the limit of the resolving power of the lens under discussion, the constant velocity distribution and voltage fluctuation of the electron beam must have ranges which together amount to less than 2 volts.

The voltage fluctuation can be reduced to this low value only by the use of large condensers and special filtering devices in the high tension system. Any existent steady velocity distribution in the electron beam is enhanced by the passage of the beam through the specimen to be examined. Direct experimental data concerning this velocity distribution is scarce. The best estimate of its magnitude can be made from the older experiments which determine the most probable velocity loss and assuming that the half-width of the velocity distribution curve is equal to the most probable velocity loss. The results of these determinations in the range of velocities of interest in this connection may be represented by a formula given by W. Bothe (11), *viz.*,

$$v_0^4 - v^4 = ax \tag{4}$$

where v_0 and v are the incident and most probable velocities, respectively, of an electron which has traversed normally a metallic film of thickness x cm. The constant a depends upon the material of the film and is related to the atomic number Z, density ρ and atomic weight A of the element composing the film by the formula given by H. Bethe (7)

$$a = k \cdot \frac{\rho}{A} \cdot Z \tag{5}$$

where k is a constant. If the velocities v_0, v are converted into the corresponding accelerating potentials $E + \Delta E$, E and the value of a for aluminium *viz.*, $a = 5.5 \times 10^{42}$, is substituted into the equations, Formulae (4) and (5) reduce to the convenient form

$$E \, . \, \Delta E = 22.1 \times 10^9 \left(\rho \frac{Z}{A} \right) x \tag{6}$$

Predictions made with this formula may be expected to hold with reasonable accuracy for values of x greater than 10^{-4} cm., for it was with films of this thickness that the underlying data of the formula were obtained. Thus it is possible to predict the magnitude of the smallest observable details in the examination of specimens which consist of sections cut by a microtome. In the present state of development of these instruments, the practical lower limit of the thickness of a cut section is about 1×10^{-4} cm. If for example, such a cut section is composed of materials the atomic properties of which are similar to those of aluminium, then by Equation (6), a velocity distribution of 580 volts is to be expected. With the use of the objective lens which possesses a resolving power of 11 Å in the examination of a specimen which introduces no chromatic aberration, such a specimen may be examined with a resolving power of only 540 Å. This does not greatly exceed the resolution attainable with a good light microscope. However, a recent publication of von Ardenne's (3), in which he claims to have developed a new microtome capable of cutting sections much thinner than the above-mentioned limit, indicates that a considerably higher resolving power will be attained in the examination of this type of specimen.

Added to chromatic aberration there is another effect arising in the object which reduces the resolving power. This is the phenomenon of angular scattering. The quantitative calculations of von Ardenne (1) concerning this effect are applicable to film thicknesses greater than 10^{-4} cm., for his calculations are based on the assumption that multiple scattering obtains in the film. However, for film thicknesses less than this by several orders of magnitude, multiple scattering does not obtain and only qualitative conclusions can be drawn. It is evident that a section of film between the object element under examination and the objective lens causes a diffusion of the electrons which is analogous to the effect of inserting a ground glass screen between an object and lens when a light microscope is employed. Since the scattering becomes more diffuse with increasing film thickness, this must be kept low on account of angular scattering as well as for the effects of chromatic aberration. Because of these two phenomena, the type of specimen which is most ideally suited to observation with the electron microscope consists of small particles that are supported on a film which can be made arbitrarily thin. In this way the form and size of a great variety of particles which are otherwise too small to be visible may be determined. It is essentially in this domain that the electron microscope will have its most useful application, for it is just the instrument required to bridge the gap between X-ray and light microscope observations.

The sources of error discussed above are of fundamental importance. There are a number of others which reduce the resolving power, but these will not be discussed further here. They are mostly of an experimental nature and their effects more amenable to control.

(c) Image Formation

It is of interest to review briefly the factors which give rise to contrast in an electron optical image, for the intensity modulation of the electron beam is caused by phenomena which differ from those causing light intensity modulation. The intensity of a point in the image is governed by the electron absorption and scattering by the material at the corresponding point in the object. The quantitative discussions of von Ardenne (1) and Marton (25) of the magnitudes of these two effects are sufficiently accurate for film thicknesses greater than 10^{-4} cm. For thinner films, scattering is the essential cause of contrast, but a quantitative discussion is impossible for the same reason mentioned in the previous paragraph. If we consider two neighbouring object elements which are of the same density but of different thickness, or of the same thickness and of different densities, the number of electrons scattered by them into a cone of given angle will differ. Since the numerical aperture of a magnetic objective is very small (10^{-2} to 10^{-3}), a slight difference in thickness or density of the two object elements produces a large difference in the number of electrons which pass through the lens to reach the corresponding image points. It has been clearly demonstrated by previous workers that specimens which must be dyed in order to be seen by means of a light microscope can be photographed with ample contrast in the image by means of an electron microscope without the use of dyes, the contrast being produced by the scattering phenomenon.

(d) Applications of the Electron Microscope

The electron microscopes of E. Ruska and L. Marton have been used to examine a large variety of specimens. Marton (26) was the first to photograph prepared biological sections. His procedure consisted in impregnating the cell structures with an osmium salt. Thereupon he photographed an osmium skeleton of the cell after the natural structure had been charred by the electron bombardment. The first unprepared biological specimens to be photographed were sections of a fly's wing. This was accomplished by H. O. Müller (16) who used Ruska's microscope. However, it was F. Krause (20, 21, p. 59) who succeeded in obtaining photographs of delicate cell structures without the use of dyeing or fixing agents.

During the summer of 1936 Krause made a thorough investigation into the sources of experimental errors of Ruska's microscope, and then made a more exact determination of the resolving power of the instrument by examining diatom structures (19, 20). He was unable to find a sufficiently fine diatom structure to determine the resolving limit, and for this reason he investigated, with the co-operation of D. Beischer (5), primary crystallites of iron and nickel aerosols in which the particle sizes had been determined independently by an X-ray method. By this means he was able to obtain evidence which led him to conclude that a resolving power of 50 Å had been reached. This investigation together with further work with D. Beischer (6) has demonstrated the usefulness of the electron microscope in colloid chemistry. The size and form of the particles of a variety of colloid systems have been determined; whereas the particle size alone could be inferred by the older established indirect methods.

One of the most interesting applications of the electron microscope up to the present has been in the examination of bacteria and viruses. Reproductions, with a previously unattained magnification of 20,400, are shown of bacteria in a publication by B. von Borries, E. Ruska and H. Ruska (10). These permitted identification of structures and particles which were unknown before. It was F. Krause (22) who first photographed viruses, the greatest dimensions of which are less than 200 Å, particles which are nearly ten times smaller than those which can be observed in a light microscope. Viruses which had been previously increased in volume by artificial means and then just detected as points in a light microscope have been photographed in their natural state at a magnification of 21,900 and seen in their true form by means of the new microscope of the Siemens and Halske Allgemeine Gesellschaft (33). In this publication, as well as in a more recent one by B. von Borries and E. Ruska (9), further excellent examples of viruses and colloidal particles at very high magnifications are shown.

Description of Apparatus

In Fig. 1 is shown schematically a cross section of the magnetic electron microscope which was constructed in this laboratory in the spring of 1938.

Electrons leaving the cathode *F* are accelerated vertically downwards and pass through the anode *A*, deflection plates *D*, and condensing lens *Co*. This lens converges the beam of electrons to a small cross section at the position of the object *O*. The object elements scatter and absorb the electrons striking them, in proportion to their relative thickness and atomic properties. The scattered electrons, which on leaving the object elements pass through the aperture of the objective lens *Obj*, are focused by the strong axially symmetrical magnetic field of this lens to conjugate image points upon the first image screen I_1. The electrons from any desired part of this image are directed through an opening in the screen into the second or projection lens *Pro*. They are focused by the magnetic field of this lens to form a magnified image of this selected part of the first image on the second screen I_2. The image at this point may be viewed with the aid of the fluorescent screen or recorded photographically by replacing the screen with a photographic plate. A high magnification is obtained in each lens by the choice of a large ratio image distance to object distance. The total magnification is of course the product of the magnifications produced by each lens.

Before various parts of this apparatus are described in more detail, some fundamental factors which were considered in its design will be discussed.

An examination of the data on the phenomena which cause aberrations in image formation shows that all of them are reduced as the accelerating potential of the electron beam is increased. Hence it is desirable to use the highest possible accelerating voltage consistent with other considerations. Ruska's

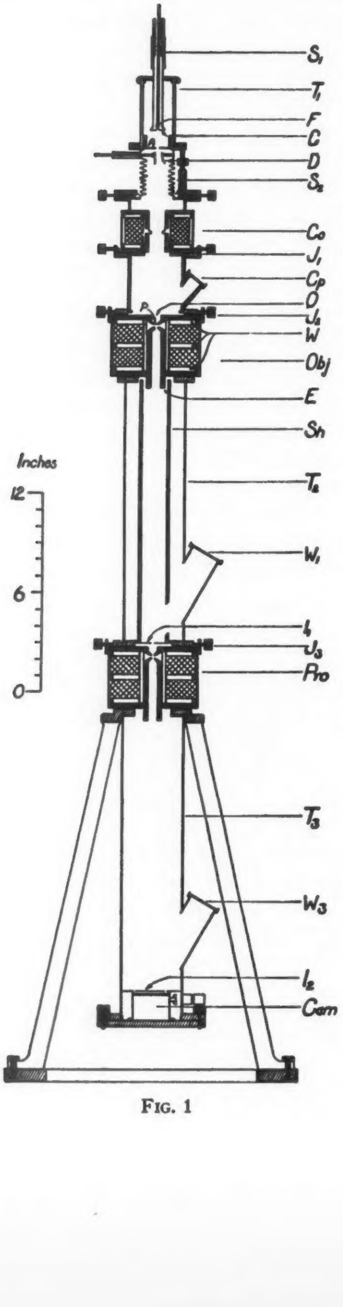
S_1
T_1
F
C
D
S_2
Co
J_1
Cp
O
J_2
W
Obj
E
Sh
T_2
W_1
I_1
J_3
Pro
T_3
W_3
I_2
Cam
Inches
12
6
0

FIG. 1

experimental results (34, 35) and von Ardenne's considerations reviewed in the "Introduction" indicate that a resolving power of the order of 10 Å might be attainable with an uncorrected simple lens. With the use of fine grained photographic plates of resolving power 1×10^{-2} mm. the minimum magnification required to record the smallest details in the image produced by such a lens is 10,000. An image distance of 50 cm. in each stage was chosen as convenient from the point of view of construction and operation. If the magnifications obtained in each stage are equal, a focal length of less than 5 mm. is required in each lens. The lenses were designed and constructed to fulfil these requirements, and later tests showed that for an accelerating potential of 45,000 volts a focal length of less than 2 mm. is attainable. Since the focal length is directly proportional to the accelerating potential of the electron beam, it appears that focal lengths of less than 5 mm. will be easily attainable at the highest potential deemed practical, *viz.*, 100,000 volts. The remainder of the instrument and the auxiliary apparatus were designed accordingly.

(a) Electron Source and High Tension System

After gas discharge tubes of various designs were tried as a source of electrons, a choice was made in favour of a hard vacuum thermionic tube because of its ease of control and constancy of operation. As shown in Fig. 1, the source of electrons is a tungsten filament F in the shape of a hairpin which is mounted at the lower end of a rod. This may be raised or lowered with respect to the remainder of the cathode assembly while the apparatus is in operation by means of a screw and bellows arrangement S_1. The lower tip of the filament is situated on the axis of the tube just above the centre of a circular opening in the concave cathode shield C. The whole cathode assembly is sealed with wax to the upper end of the glass tube T_1 which serves as an insulator. The adjustment of the filament position with respect to the cathode shield overcomes two difficulties found in the use of a hot cathode as an electron source. First, it is possible to draw the filament far enough into the cathode shield so that only the electrons emitted from the very tip of the hairpin filament can escape. Thus the advantage of point emission of the gas discharge tube is retained. Second, the efficiency of the thermionic cathode is greatly increased, and it is possible to keep the total emission current well below the limit set by the permissible chromatic error with ample intensity of the image for observation. In the high tension system used, the voltage ripple and consequent chromatic error increases directly with the total emission current.

The high tension system consists of a step-up transformer with one terminal earthed, hard vacuum half-wave rectifier, and a filter system. This consists of a condenser the negative terminal of which is connected to the anode of the rectifier and the cathode of a saturation diode, the anode of which is connected to the negative terminal of a second condenser. The cathode of the microscope discharge tube is connected through a stabilizing resistance to the negative terminal of this second condenser. All of the high tension apparatus and connecting leads are carefully shielded against the effects of corona. Calculation shows that if the total current drawn is below 0.1 ma. the fluctuation in the accelerating potential is less than 1 volt in 50,000. The actual control of the ripple is highly sensitive to the adjustment of the saturation diode.

(b) Beam Trap and Condenser Lens

The beam trap consists of a pair of deflection plates *D*, Fig. 1, so arranged that the beam is deflected away from the object when one plate is raised to a potential of 2,000 volts with respect to the other. It is used as a shutter when a photographic exposure is made and in general to limit the time of exposure of the object to the electron beam to the minimum which is required for focusing and photographing.

The electron source and the beam trap are attached to the upper end of the condenser by means of a bellows adjustment arrangement S_2 which allows the source to be displaced horizontally or tilted in any direction in order to bring the electron beam into coincidence with the axis of the condenser coil. The condenser *Co* is an iron encased coil with a central circular gap which concentrates the magnetic field to a small region along its axis. The coil consists of 2700 turns of No. 24 B & S enamelled wire and it is provided with a disc shaped water cooling jacket on each side. The lower face of the iron casing is ground plane and sealed with vacuum grease to a corresponding ground surface J_1 on the upper side of the object chamber. By means of this sliding joint and adjustment screws it is possible to move the electron source, beam trap, and condenser as a unit in any direction perpendicular to the axis of the microscope. In this way the illuminating pencil of electrons can be brought into coincidence with the axis of the objective lens, and the image of the emitting area of the cathode can be focused upon the centre of the object.

(c) Object Chamber and Specimen Holders

Specimen holders are inserted into the apparatus through the opening *Cp* of the object chamber. This opening is a simple conical grease joint. Two types of specimen holders have been used. One of these is in the form of a hollow cartridge which fits snugly into a collar over the objective lens. The lower end of the cartridge is about 5 mm. above the centre of the gap between the pole pieces of the lens. This simple arrangement makes it easy to replace the specimen holder into exactly the same position after it has been removed and reloaded. The other type of specimen holder is a similar cartridge mounted on the end of a hollow screw. With this it has been possible to vary the object distance during operation by turning the plug of a grease joint to which the screw is geared. This joint is fixed in the wall of the object chamber. The actual specimens are suspended across a small hole (0.05 to 0.3 mm.) in the centre of a circular platinum diaphragm which is held at the lower end of the specimen holder by a cap. During operation the holders are held in position by a support fixed in the object chamber. Since this is sealed to the objective lens by means of a flat grease joint J_2, the object is readily displaced in any horizontal direction. Hence any part of it may be selected for examination.

(d) Lenses

In the design of the lenses, the foremost consideration was given to ease of adaptability or alteration, for it was expected that their optical properties would vary widely with the configuration and strength of their fields. Since, with the exception of the pole pieces, the projection coil is identical with the objective, a description of only one of them will be given.

Two coils, each of 2700 turns of No. 22 B & S enamelled wire are wound upon a brass cylinder. To the ends of this cylinder are soldered two iron discs with their outer faces ground plane. Similar ground plates forming the bases of the image tubes and object chamber may be sealed to the coil with vacuum grease. The outer iron cylinder which slides snugly over the coil, and the inner sleeve *E*, which is threaded into the base plate and central brass cylinder, complete the magnetic circuit. Into the upper end plate and upper end of the central sleeve, interchangeable pole pieces *P* are threaded. The distance between these may be altered at will by turning the sleeve *E*. Three disc shaped water jackets *W* are provided for cooling the coils and the grease joints.

The pole pieces in use are conical with flattened ends. An accurately machined brass spacer arrangement placed between the pole pieces permits them to be aligned with precision and fixed rigidly. The diaphragm which is used to limit the spherical aberration of the lens is a piece of platinum foil through which a small hole (0.1 to 0.05 mm. in diameter) is punched. This is soldered to the end of a brass tube which fits tightly inside the spacer.

(e) Miscellaneous Parts

At the lower end of the brass tube T_2, which separates the objective and projection lenses, a fluorescent screen is mounted upon which the first image may be viewed through the window W_1. Inside this first tube and concentric with it there is a soft iron pipe *Sh* which is used to shield the electron beam from stray varying magnetic fields. The third grease joint J_3 allows the second image to be displaced relative to the projection coil. The second tube in the microscope T_3 is fastened to the support of the instrument. This consists of four bronze legs. The lower end of the tube carries the camera *Cam* and the second image screen I_2 which are fastened to the upper side of the plate that seals the end of the tube. This also is a flat grease joint. The camera consists of a platform which bears the photographic plate and a hinged fluorescent screen I_2 which covers it. When a photographic record of the image is desired, the screen may be lifted out of the way by means of a conical grease joint fixed in the wall of the tube.

Experimental Results

The work carried out with the apparatus since its construction was completed has been restricted to the identification and elimination of the perturbing effects which cause undesired movements of the image. These effects are numerous and have not yet been overcome completely.

As mentioned under a previous heading, a cold cathode gas discharge tube was used initially as the source of the electron beam. This was chosen because of two advantages which it possesses over a hard vacuum thermionic cathode tube. Emission of the electrons from a small spot of the cathode allows a beam of small cross section to be focussed upon the object without the necessity of a specially constructed electron converging lens. Secondly, the impedance of a gas discharge tube to a voltage ripple in the high tension system is an order of magnitude lower than that of a thermionic cathode tube. This property of the tube was considered as a necessary complement to the filter system initially employed. However, all the various gas discharge tubes which were tried were found defective in one respect. Subsequent to the letting of air into the apparatus, they required several hours of operation before they reached a steady state. The delays occasioned by this troublesome outgassing process prevented a systematic study of other perturbing effects. An efficient thermionic cathode tube and magnetic condensing lens were finally constructed, and with these it is possible to obtain steady operating conditions immediately after the apparatus has been sufficiently well exhausted. Furthermore, the intensity of the electron beam can be varied within wide limits, and it is now possible to observe with ease images magnified 20,000 diameters and to photograph them in exposure times of the order of one second.

Another of the major difficulties encountered is the effect of electric charges which are built up on the surfaces of the specimen holders and lens diaphragms owing to oxidation brought about by electron bombardment and subsequent exposure to air. It was found necessary to shield all parts of the specimen holders, lens diaphragms, and spacers from exposure to the electron beam by means of platinum diaphragms which can readily be removed and cleaned.

Destruction of the collodion membranes which are used to support the specimens by the heating effect of the electron beam was also a cause of trouble. A thin film suspended across the opening of a diaphragm should be heated in proportion to its absorption coefficient for the electron beam. However it was found that quite transparent membranes ruptured and disintegrated rapidly when under observation, apparently because of excessive heating. The cause of this was traced to the absorption of electrons by the metal diaphragm over which the membrane was suspended. By placing a

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CAPTIONS FOR PLATE I, FACING THIS PAGE.

FIG. 2-A. *Light microscope photograph of Pleurosigma angulatum; magnification, 480; 4 mm. water immersion achromatic objective of N. A. 1.00; blue filter.*

FIG. 2-B. *Light microscope photograph of Pleurosigma angulatum; magnification, 1070; 1/12 in. oil immersion achromatic objective of N. A. 1.30; blue filter.*

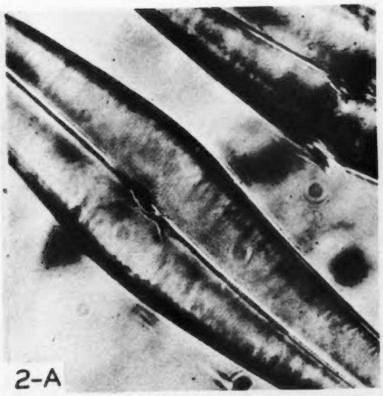
FIG. 2-C. *Electron microscope photograph of Pleurosigma angulatum; magnification, 1700; enlarged to 2500. Accelerating potential, 16 kv.*

FIG. 2-D. *Electron microscope photograph of Pleurosigma angulatum; magnification, 6250; enlarged to 25,000. Accelerating potential, 45 kv.*

FIG. 2-E. *Electron microscope photograph of Pleurosigma angulatum; magnification, 9000; enlarged to 36,000. Accelerating potential, 45 kv.*

FIG. 2-F. *Electron microscope photograph of edge of melted section of Pleurosigma angulatum; magnification, 20,000; enlarged to 80,000. Accelerating potential, 45 kv.*

PLATE I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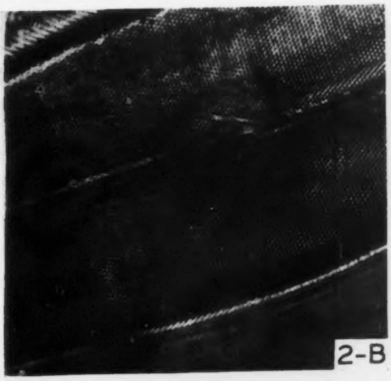
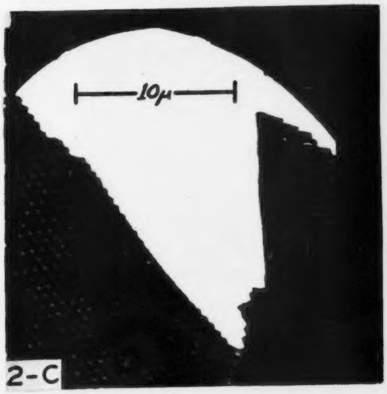
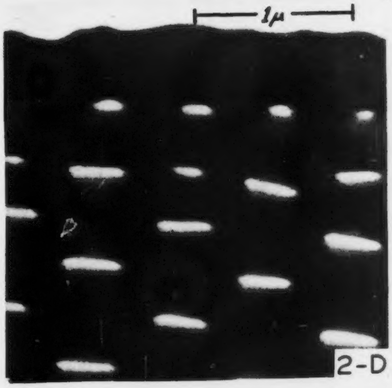
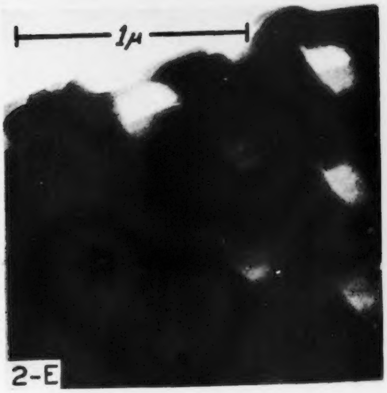

2-B



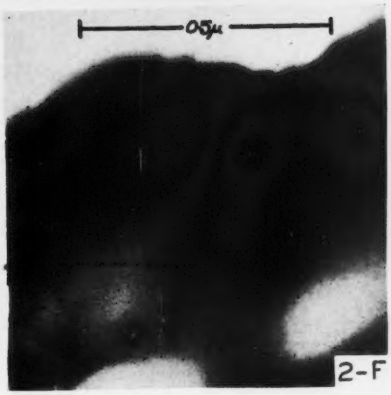
2-C



2-D



2-E



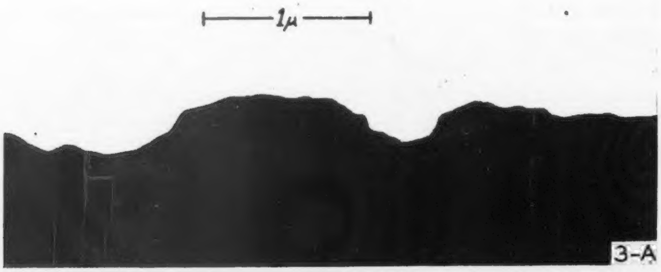
2-F

diaphragm a considerable distance above the specimen to limit the cross section of the electron beam so that it struck the membrane alone, the heat generated by the absorption of electrons by the metal was dissipated by conduction away from the specimen.

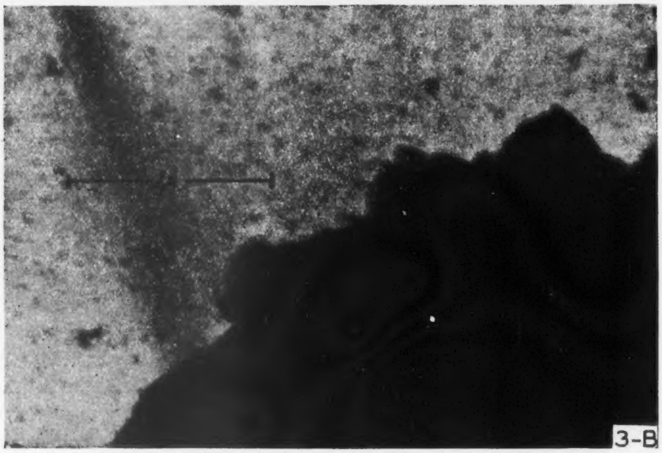
The photographs shown in Figs. 2 and 3 are selected from those which have been taken in the course of the investigation of the effects which impair the quality of the photographic records of the observed images.

Figs. 2-A and 2-B are light microscope photographs of sections of a diatom *(Pleurosigma angulatum)* magnified 480 and 1070 diameters respectively. These are representative of the resolving power attainable with a good microscope. Fig. 2-A was taken with the use of a water immersion achromatic objective lens of focal length 4 mm. and numerical aperture 1.00 with blue light illumination; Fig. 2-B with an achromatic 1/12 in. oil immersion objective of numerical aperture 1.30. Fig. 2-C is a reproduction of a section of the same diatom magnified 2500 diameters; this was obtained at a magnification of 1700 diameters with the electron microscope shortly after its completion. The accelerating potential of the electron beam was 16 kv. Although a shift of the image caused by an unavoidable change of pressure in the discharge tube then used is evident, the sharpest bounding edge of the diatom in the photograph allows a resolving power of 600 Å to be inferred. This is considerably better than the resolving power shown in the light microscope photographs. Figs. 2-D, 2-E, and 2-F are reproductions, enlarged four diameters, of electron microscope images of the same type of diatom which have been obtained recently. These are magnified 6,250, 9,000, and 20,000 diameters respectively by the electron microscope. Although the photographs demonstrate the high resolving power attained with the apparatus, an exact evaluation of its magnitude cannot be made from them owing to the fact that the bounding edges of the structures are not sharply defined for a high velocity electron beam. In Fig. 2-E the effect of an image shift is again evident. Fig. 2-F demonstrates the sharpness of image definition attainable even at the high magnification of 20,000 diameters. It shows a small section of the silica shell of a diatom after it has been partially melted by the heat of the electron beam.

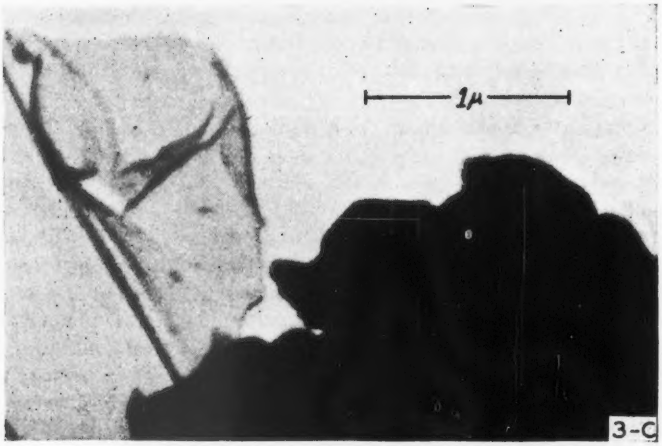
Fig. 3-A is an enlarged reproduction (four diameters) of the image of the cutting edge of an unused razor blade magnified 8,000 diameters by means of the electron microscope; double the width of the best defined regions of the bounding edge, as measured in the original of the reproduction, divided by the corresponding magnification, leads to a value of 140 Å for the resolving power shown in this photograph. Figs. 3-B and 3-C are similarly enlarged reproductions of images, magnified 9,000 diameters by the electron microscope, of particles of two specimens of clay supported on a collodion membrane approximately 150 Å thick. These specimens are of a fresh and burned clay, respectively, which is used in refining gasoline. The photographs are also of interest because of the large number of small particles visible, whose origin is in the supporting membrane. A number of pairs of these particles may be



3-A



3-B



3-C

found which are just separated. The smallest distances of separation, measured in the original, show that the resolving power is better than 200 Å.

Acknowledgments

The authors take this opportunity to express their appreciation to Dr. E. F. Burton, Director of the McLennan Laboratory, University of Toronto, for the interest he has shown in the progress of this work and for the laboratory facilities which he has placed at their disposal.

To Prof. Stanley Smith, Department of Physics, University of Alberta, they wish to express their gratitude for the loan of two high tension condensers which are the property of the University of Alberta.

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CAPTIONS FOR FIGS. 3-A, 3-B, AND 3-C, FACING THIS PAGE.
(Reproductions slightly reduced in printing)

FIG. 3-A. *Electron microscope photograph of the cutting edge of a razor blade; magnification, 8000; enlarged to 32,000. Accelerating potential, 45 kv.*

FIG. 3-B. *Electron microscope photograph of a particle of fresh clay. Blackened area is a section of the particle; background is the supporting collodion membrane; magnification, 9000; enlarged to 36,000; Accelerating potential, 45 kv.*

FIG. 3-C. *Electron microscope photograph of a particle of burned clay. Blackened area is a section of the particle. The ruptured supporting membrane is shown at the left; magnification, 9000; enlarged to 36,000. Accelerating potential, 45 kv.*

THE EFFECT OF CHROMATIC ERROR ON ELECTRON MICROSCOPE IMAGES[1]

BY JAMES HILLIER[2]

Abstract

The effect on the intensity distribution of point to point variation of the chromatic error in electron microscope images is investigated qualitatively. The intensity distribution in the image is found to possess a maximum value just outside, and a minimum value just inside, the image of the edge of the object. The deductions are verified by microphotometer traces taken across the photographic record of the image of a diatom edge. It is suggested that the intensity distribution curve may be used in the physical analysis of the object.

Owing to the close analogy between geometrical electron optics and geometrical light optics it is possible to apply the methods of the latter to the investigation of the errors present in the images produced by electron lenses. The large amount of theoretical and experimental work which has been done on this subject during the past few years has justified the use of these methods. However, the *variation* in the chromatic error from point to point in the image of an inhomogeneous object (of varying thickness and density) has no counterpart in light optical phenomena. In the following work, the effect of this variation on the intensity distribution in the image is considered from a purely qualitative point of view.

The electron intensity distribution in the image of an ideal test object, which consists of a film of some organic material in the position of the focal plane of the electron microscope objective, will be considered. In order to introduce a variation in the chromatic error present in the image of this film it will be assumed that the film in half of the field of view of the objective is uniform and extremely thin, while that in the other half is also uniform but thicker and of denser material. This object is an ideal representation of the usual type of biological specimen viewed by the electron microscope, *viz.*, one in which the organisms under investigation are supported on an extremely thin carrier film of collodion. Furthermore, it is assumed that the incident electrons are travelling parallel to the axis of the objective and with equal velocities. Fig. 1 is a diagrammatic representation of the cross section of such an arrangement.

In passing through an element of area of a thin film, electrons may suffer a number of elastic and inelastic collisions with the atoms composing the film, so that, on emergence, their direction of motion may be altered. Those

AUTHOR'S NOTE:

This paper is the result of a discussion with Prof. C. D. Ellis during his recent stay in Toronto. Professor Ellis suggested that the chromatic error may be used in the physical interpretation of the images obtained with the electron microscope.

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[1] *Manuscript received February 13, 1939.*

Contribution from the McLennan Laboratory, Department of Physics, University of Toronto, Toronto, Canada.

[2] *Demonstrator, Department of Physics, University of Toronto.*

electrons with directions outside the cone subtended at the point of emergence by the aperture of the objective do not pass through the aperture and thus are prevented from reaching the image. The probability of an incident electron being deflected from its path by the effect of the relatively high charge on the atomic nuclei is small but, nevertheless, appreciable for the thin part of the film and very large for the thick part. As a result, a larger number of the electrons leaving the thick part of the film are scattered outside the aperture of the objective. Furthermore, the emergent electrons may possess different velocities which are, in general, less than the homogeneous velocity of the incident beam. In the case of a film which is only a few atoms thick the probability of the occurrence of a sufficient number of inelastic collisions between a high speed incident electron and the atomic electrons to cause an appreciable lowering of the velocity of the electron is extremely small. However, as this probability increases rapidly with thickness and density, the electrons emerging from the thicker film possess a wide range of velocities.

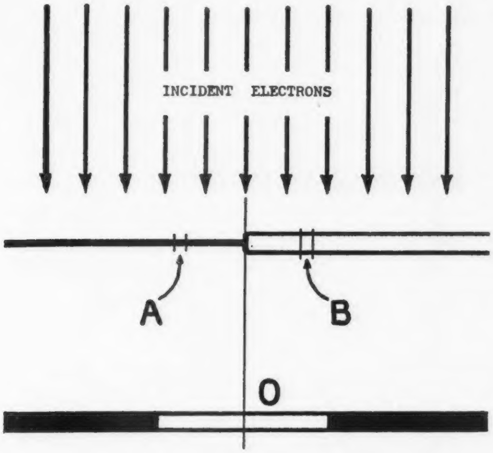

FIG. 1

If the objective lens were perfect the intensity distribution in the image of a small element of area at *A* (Fig. 1) in the thin part of the film would be represented by the curve shown in Fig. 2*a*. The area under the curve is a representation of the number of electrons which reach the image after passing through the element of area at *A* and the aperture of the objective *O*. As shown above, the chromatic aberration is negligible in the case of the thin film; however, owing to spherical aberration, there is a small image error present which modifies this curve so that it has a shape similar to that of a narrow error function curve as shown in Fig. 2*b*. As the spherical aberration has no effect on the number of electrons reaching the image, the area under the curve in Fig. 2*b* is the same as that under the curve in Fig. 2*a*. Once again, if the objective lens were perfect the intensity distribution in the image of an element of area at *B* (Fig. 1) in the thick film would be represented by the curve shown in Fig. 2*c*. The difference in the intensities of the images of elements of area at *A* and *B* (*i.e.*, the contrast) introduced by the spatial scattering is represented by the relative areas under the curves in Figs. 2*a* and 2*c*. However, owing to the effect of chromatic as well as spherical aberrations in the image of an element of area at *B*, the intensity distribution curve obtained in practice has a shape very similar to that of a very broad error function as shown in Fig. 2*d*. The area under the curve in Fig. 2*d* is equal to that under the curve in Fig. 2*c*.

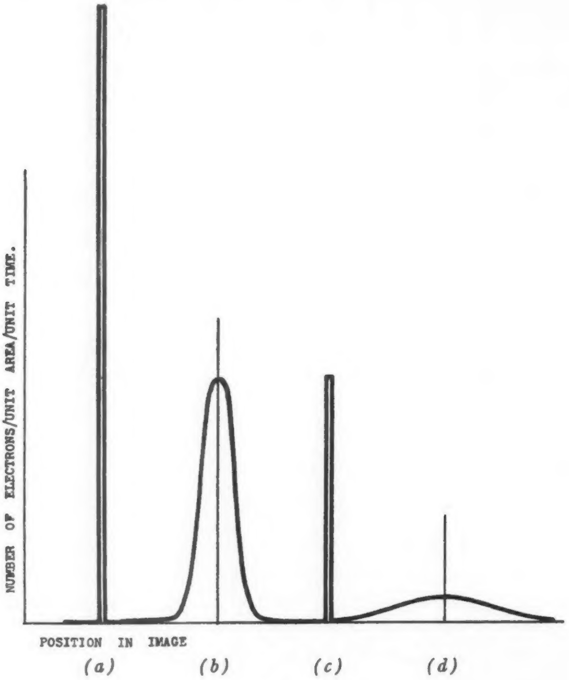

FIG. 2

The intensity distribution in the entire image will now be determined by superposing the images of all the elements of area of the object shown in Fig. 1. Since, in the image of any object element, there is a measurable intensity over a considerably larger area than that of the geometrical image, the intensity at any one point of the image will depend not only on the number of electrons reaching it from the corresponding object point but also on the number reaching it from all other points of the object. This is represented graphically in Fig. 3, where the curve of the intensity distribution in the image

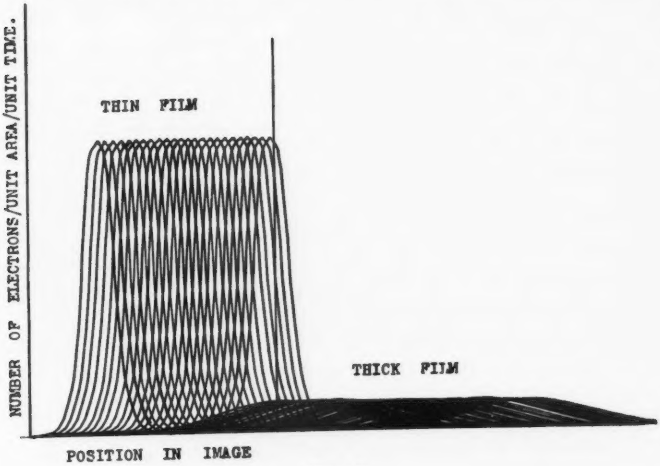
NUMBER OF ELECTRONS/UNIT AREA/UNIT TIME.
THIN FILM
THICK FILM
POSITION IN IMAGE

FIG. 3

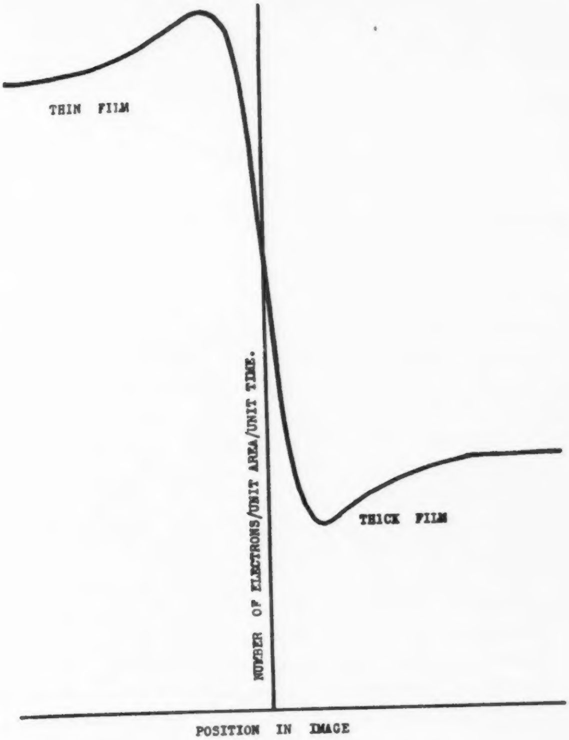
THIN FILM
NUMBER OF ELECTRONS/UNIT AREA/UNIT TIME.
THICK FILM
POSITION IN IMAGE

FIG. 4

of each element of area is drawn in its proper position. The intensity at any point in the image is determined by the sum of the ordinates (taken at the point in question) of all the curves which have a measurable value at the point. The actual intensity distribution in the complete image may then be plotted. This has been done in Fig. 4, and gives a rather unexpected result that has no analogue in the light optical theory of images. Just outside the geometrical image of the edge of the thick film there is a maximum in which the intensity is greater than in the image of the thin film, while just inside this edge there is a minimum in which the intensity is less than in the image of the thick film. Moreover, the shape of this intensity distribution depends on the difference in the magnitude and nature of the angular and velocity distributions of electrons leaving the two parts of the object.

In practice, this means that a band of greater intensity than that in the image of the supporting film should appear just outside the geometrical image of the edge of the organism under investigation, while a band of lower intensity than that in the image of the organism should appear just inside this edge. A number of photographs which have been taken with the electron microscope described in the preceding paper show this effect very clearly. Fig. 5 is a reproduction of a microphotometer trace taken across the photographic record of the image of a diatom edge. This organism has a maximum thickness of about 0.5 μ which decreases considerably towards the edge and is, therefore, sufficiently transparent to electrons for this test. In Fig. 6 the photographic blackening has been plotted from the curve in Fig. 5. As the blackening is approximately proportional to the electron intensity, this curve represents the actual intensity in the electron microscope image. The fact that the minimum intensity is not as clearly defined as in the hypothetical case may be due to the fact that thickness of the diatom is increasing at a short distance from the edge. This is indicated by the decrease in intensity as one proceeds farther into the image of the diatom.

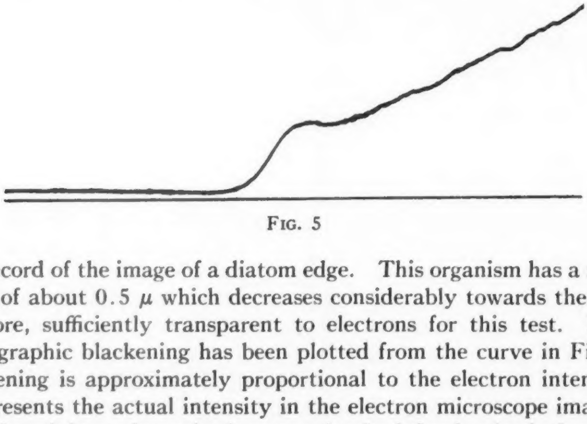

FIG. 5

From the above considerations, it is apparent that the contrast in electron microscope images is due to the differences in spatial scattering of the electrons by the object elements, while the unusual intensity distribution in the images of boundary edges is entirely due to the electron velocity distribution which has been introduced by the material of the object. In other words, the physical interaction between a beam of electrons and the object produces two measurable, independent image effects which have no counterpart in light optics. A complete quantitative study of these effects, which the author hopes to make the subject of a future paper, should lead to a more precise physical interpretation of electron microscope images.

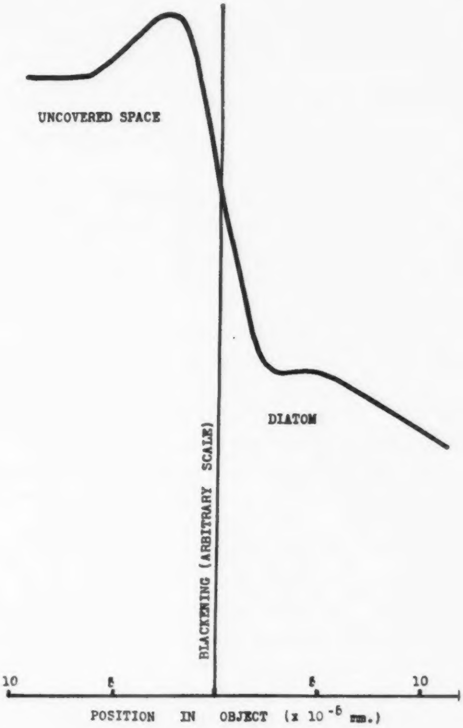

FIG. 6

Acknowledgments

The author wishes to thank Prof. C. D. Ellis for his suggestions, and to express his appreciation of the interest in this work shown by Prof. E. F. Burton. The photographs used were obtained by Mr. A. Prebus.

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A STUDY OF THE MECHANISM AND KINETICS OF THE SULPHITE PROCESS[1]

BY J. M. CALHOUN[2], F. H. YORSTON[3], AND O. MAASS[4]

Abstract

The rate of delignification of resin extracted spruce wood-meal has been determined in calcium-base sulphite liquor at temperatures from 130° C. down to 50° C. No break was found in the temperature coefficient curve at the lower temperatures, the reaction following the Arrhenius equation closely. Possible mechanisms of the reaction are discussed in the light of existing theories, and the effect of temperature on the yield of pulp is pointed out for its practical interest.

Introduction

A study of the kinetics of a chemical reaction is usually made by measuring the reaction velocity at different temperatures and at different initial concentrations of each reactant over as wide a range as possible. In the case of the delignification of wood in sulphite liquor, the problem is complicated by the large number of variable factors involved and by the heterogeneous nature of the reaction. Nevertheless, it was felt that an investigation of the effect of liquor composition and temperature on the rate of delignification would throw some light on the mechanism and kinetics of the reaction.

The variation in the rate of delignification of spruce wood with the liquor composition has previously been determined and reported by the authors, both for calcium-base (1) and magnesium-base liquor (2). With regard to the variation in reaction rate with temperature, very little quantitative information is available. Stangeland (11) made some small-scale laboratory cooks and reported that the rate of removal of "incrustants" (all materials other than Cross and Bevan cellulose) was approximately first order, and that the temperature coefficient was close to 2.0 per 10° interval between 110° and 140° C. Yorston (12, 13) found that the rate of removal of lignin showed a systematic deviation from the first order relation. Corey and Maass (3) cooked two samples of wood-meal at several temperatures, and assuming a first order relation showed that the variation in reaction rate with temperature followed the Arrhenius equation between 100° and 140° C.

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Contribution from the Department of Physical Chemistry, McGill University, Montreal, Canada. This investigation was carried out in co-operation with the Forest Products Laboratories of Canada, Montreal, and formed part of the research program of that institution.

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[3] *Chemist, Forest Products Laboratories, Montreal, Department of Mines and Resources.*

[4] *Macdonald Professor of Physical Chemistry, McGill University, Montreal, Canada.*

It was desired to repeat the latter work more accurately and over a greater temperature range. Particular interest lay, for several reasons, in low temperature cooks. It has been a common belief in the sulphite industry that wood cannot be satisfactorily delignified below 110° C., and Sankey and Hibbert (10) have stated that the sulphonation reaction does not take place below this temperature. If this were true, a change in the slope of the temperature coefficient curve would be expected; this might give a clue to the mechanism of the reaction. A quantitative study of the effect of temperature on the yield of pulp is of both theoretical and practical interest.

Experimental

The experimental procedure followed was identical in every detail with that previously described for the determination of the effect of liquor composition (1). Well seasoned, white spruce wood-meal (density, 0.43; mesh, 40 to 100) was prepared from the same log and extracted with alcohol benzene (1 : 2). The lignin content was 27.5%. All cooks were carried out in 100 cc. sealed Pyrex bomb tubes with calcium-base sulphite liquor containing initially about 1.0% combined, and 10% free, sulphur dioxide. The liquor ratio (50 : 1) was sufficiently large to minimize any concentration changes during cooking. Six samples of wood-meal were cooked for different lengths of time at each temperature in an oil bath thermostatically controlled to within 0.1° C.

The mean liquor concentration for a given run was taken as the average of that for all the bombs, analysis being made after cooking. The liquor concentration as determined at room temperature was corrected, in the manner previously described, for the sulphur dioxide lost to the vapour phase at cooking temperatures. The observed time of cooking was that recorded from the moment the bombs were dropped into the bath until they were quenched in water. A correction for the time required to heat the bombs to the cooking temperature, in terms of the time required to do the same amount of cooking at that temperature, was determined and applied as described before. This amounted to −0.22 hr. at 130° C., −0.18 hr. at 110° C., and was insignificant at lower temperatures. The cooked wood-meal was filtered, washed, oven dried, weighed, and analyzed for lignin by the Ross-Potter method (9). All yields were calculated on the resin extracted, bone-dry wood basis.

Results

The Effect of Temperature on the Yield of Pulp

Cooks were made at 50°, 70°, 90°, 110°, and 130° C.; the results are given in Table I. Since pulp yields must be compared on the basis of the same lignin content, the effect of temperature on yield can be most clearly shown by plotting the yield of pulp against the percentage lignin removed in each cook. This has been done in Fig. 1, and it is observed that the yield of pulp at any given lignin content decreases with increase in the cooking temperature. The yields of pulp at 50, 80, 90 and 95% delignification were determined from

TABLE I

THE EFFECT OF TEMPERATURE ON THE RATE OF DELIGNIFICATION OF SPRUCE WOOD AND YIELD OF PULP

I	II	III	IV	V	VI	VII	VIII
Time in bath (uncorrected), hr.	Combined sulphur dioxide, %	Total sulphur dioxide (uncorrected), %	Total sulphur dioxide (corrected) %	Yield of pulp, %	Yield of non-lignin, %	Lignin, per cent of pulp	Lignin, per cent of original wood
Temperature, 50° C.							
150	0.95	10.40	10.40	91.6	74.4	18.8	17.2
310	0.95	10.20	10.20	87.8	75.7	13.8	12.1
500	0.95	10.50	10.50	85.6	77.1	9.91	8.48
910	0.98	10.50	10.50	78.7	76.3	2.98	2.35
1510	0.92	10.30	10.30	73.5	72.6	1.23	0.90
Average	0.96	10.38	10.38				
Temperature, 70° C.							
36	0.97	11.08	11.08	87.1	71.1	18.4	16.0
72	0.94	10.96	10.96	81.6	71.7	12.2	9.91
96	0.94	10.96	10.96	78.8	72.3	8.14	6.46
120	0.92	10.78	10.78	75.3	70.5	6.31	4.75
144	0.92	10.72	10.72	72.3	69.6	3.76	2.72
180	0.90	10.58	10.58	69.3	67.8	2.10	1.45
216	0.94	10.82	10.82	66.8	65.9	1.34	0.90
Average	0.93	10.84	10.84				
Temperature, 90° C.							
12.0	0.98	10.88	10.87	79.3	69.1	12.9	10.2
24.0	0.95	10.84	10.82	69.0	66.0	4.39	3.03
36.0	0.94	10.88	10.85	62.2	61.0	1.97	1.23
48.2	0.95	10.68	10.66	59.3	58.5	1.26	0.75
Average	0.95	10.82	10.80				
Temperature, 110° C.							
2.00	0.94	10.60	10.55	78.7	62.8	20.2	15.9
4.00	0.94	10.60	10.53	69.7	59.9	14.0	9.77
5.50	0.90	10.72	10.62	64.6	59.5	7.97	5.14
8.00	0.90	10.88	10.80	57.5	55.8	2.97	1.71
10.00	0.90	10.92	10.82	54.4	53.6	1.47	0.80
Average	0.91	10.74	10.66				
Temperature, 130° C.							
0.50	0.96	11.06	10.90	81.0	61.4	54.2	19.6
1.00	0.92	11.06	10.92	69.6	57.2	17.8	12.4
1.50	0.90	10.88	10.75	62.0	55.2	10.96	6.80
2.00	0.83	10.72	10.59	55.7	52.9	4.96	2.76
2.50	0.84	10.80	10.61	52.1	50.8	2.55	1.33
3.00	0.78	10.36	10.15	50.4	49.4	1.93	0.97
Average	0.87	10.81	10.65				

these curves and replotted against the temperature in Fig. 2. The horizontal dotted lines represent the maximum yields possible, *i.e.*, the sum of the lignin plus the original non-lignin content of the wood. The actual yields approach their maximum values below 70° C. That is, very little or no carbohydrate material is removed from the wood at these lower temperatures.

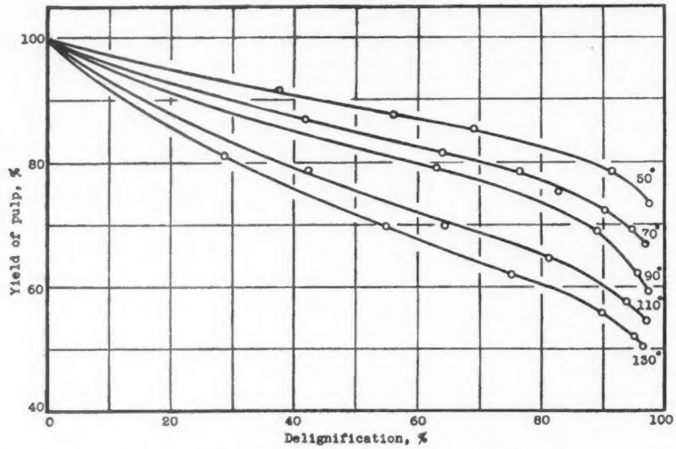

FIG. 1. *The effect of temperature on the yield of pulp.*

It should be mentioned that the yields of non-lignin reported at 50°C. in Table I appear to be anomalous, as they are slightly higher than the non-lignin content of the original wood (72.5%). The only explanation would seem to be that the lignin values are in error, because the non-lignin is obtained by difference. However, the lignin analysis was done in duplicate for this particular run and good checks were obtained.*

The curves in Fig. 2 show a slight tendency to flatten out at the higher temperatures. This is to be expected since some of the carbohydrate material is much more resistant than the remainder. Nevertheless it is clear that temperatures of 150° C., which are sometimes reached in commercial pulping, would be extremely harmful from the point of view of yield. The practical advantage of very low cooking temperatures in increased yields of pulp is offset by the long time required for cooking. However, an intermediate cooking temperature of, say, 90° C., using a liquor high in free sulphur dioxide, might result in an improvement in quality as well as a high yield.

* *Since the above was written, J. Cannon, in this laboratory, has determined holocellulose, chlorine number, and lignin by the 72% sulphuric acid method in pulps cooked at 70°, 100°, and 130° C. The delignification at the lowest temperature, as judged by these analyses, is less than that indicated by the Ross-Potter method. Temperature coefficients of the pulping reaction based on chlorine number or on residual lignin as determined by 72% sulphuric acid appear to be about 5% greater than the coefficient calculated from analyses by the Ross-Potter method. The discrepancy seems to be due to a peculiarity of the sulphonated lignin in pulp cooked at a very low temperature; such lignin is slightly soluble under the conditions of the Ross-Potter analysis.*

The Temperature Coefficient of Delignification

The lignin remaining in the pulp after each cook, calculated as a percentage of the original wood, is plotted on a logarithmic scale against the observed time of cooking in Fig. 3. These delignification curves are all similar in shape and show the same deviation from the straight line, first order relation reported by Yorston (12) and others (1, 2, 3). The curve obtained at 50° C. proves that wood can be delignified by the sulphite process at this temperature, a fact that is contrary to general belief. This statement is true, regardless of possible small errors in lignin analysis for this run, which would only slightly alter the shape of the curve. However, a reservation must be made regarding the validity of the velocity constants calculated for this run until further investigation has been made.

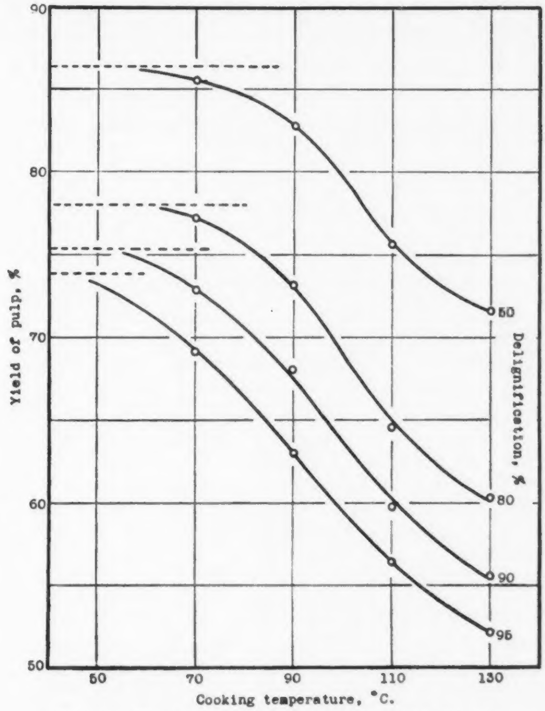

FIG. 2. *The effect of temperature on the yield of pulp.*

The calculation of the temperature coefficient of delignification is complicated by the deviation of the reaction from the first order relation, since an estimation of velocity constants is necessary. However, the following method was adopted. The observed times required to bring about 50, 80, 90, and 95% delignification at each temperature were read from the curves in Fig. 3; these are listed in Table II. The time correction was then applied for the cooks made at 110° and 130° C. by subtracting 0.18 and 0.22 hr. respectively. Finally, all the times were corrected to correspond to cooking with the same concentration of free sulphur dioxide (9.75%). This was necessary because it was impossible to predetermine the exact liquor composition, and small variations resulted from run to run. This correction was readily made since the authors have shown (1) that the time of cooking at constant combined, is inversely proportional to the concentration of free, sulphur dioxide, at least over short ranges.

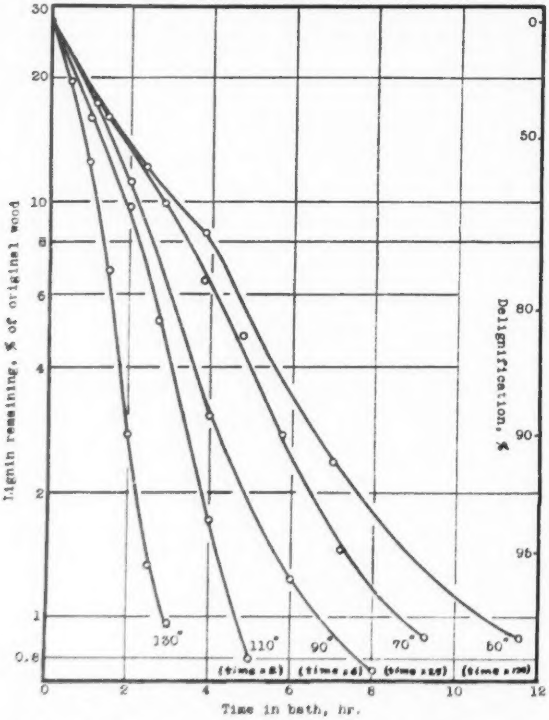

FIG. 3. *The effect of temperature on the rate of delignification.*

First order velocity constants were then calculated for the ranges 0 to 50%, 50 to 80%, 80 to 90%, 90 to 95% delignification, all expressed in units of seconds. Although these velocity constants vary at different stages in the cooking process at any one temperature as expected, it is believed that the mean velocity constants calculated in this manner represent as fair a value as it is possible to obtain.

TABLE II

CALCULATION OF VELOCITY CONSTANTS AND THE TEMPERATURE COEFFICIENT OF DELIGNIFICATION

Temperature, °C.	50	70	90	110	130
Average concentration of sulphur dioxide, % (corrected)					
Total	10.38	10.85	10.80	10.66	10.65
Combined	0.96	0.93	0.95	0.91	0.87
Free	9.42	9.92	9.85	9.75	9.78
Time, hr. (observed)					
To 50% delignification	245	48	8.80	2.59	0.90
To 80% delignification	655	109	18.4	5.36	1.64
To 90% delignification	855	143	25.1	6.80	2.00
To 95% delignification	1180	184	33.8	8.45	2.47
Time, hr. (corrected for time required to reach temperature)					
To 50% delignification	245	48	8.80	2.41	0.68
To 80% delignification	655	109	18.4	5.18	1.42
To 90% delignification	855	143	25.1	6.62	1.78
To 95% delignification	1180	184	33.8	8.27	2.25
Time, hr. (corrected to 9.75% free sulphur dioxide)					
To 50% delignification	237	48.8	8.88	2.41	0.68
To 80% delignification	632	111	18.6	5.18	1.42
To 90% delignification	826	145	25.3	6.62	1.78
To 95% delignification	1140	187	34.1	8.27	2.25
First order velocity constants, $k \times 10^5$ (sec.$^{-1}$)					
0–50% delignification	0.0813	0.395	2.17	7.99	28.3
50–80% delignification	0.0644	0.410	2.62	9.19	34.4
80–90% delignification	0.0997	0.567	2.87	13.37	53.5
90–95% delignification	0.0613	0.459	2.19	11.67	41.0
Average	0.0767	0.458	2.46	10.56	39.3
Temperature coefficient per 10° C.	2.44	2.32	2.07	1.93	

The approximate temperature coefficient per 10 degrees was calculated for each interval according to the formula

$$\left(\frac{k_2}{k_1}\right)^{\frac{t_2-t_1}{10}}, \tag{1}$$

where k_2 = the velocity constant at the higher temperature, t_2° C.,

k_1 = the velocity constant at the lower temperature, t_1° C.

The calculated values shown at the bottom of Table II are close to 2, which is in agreement with results given in previous reports (3, 11).

If the integrated form of the Arrhenius equation

$$\ln k = \ln Z - E/RT, \qquad (2)$$

where k = the velocity constant,
T = the absolute temperature,
R = the molar gas constant,
Z, E = specific constants for each reaction,

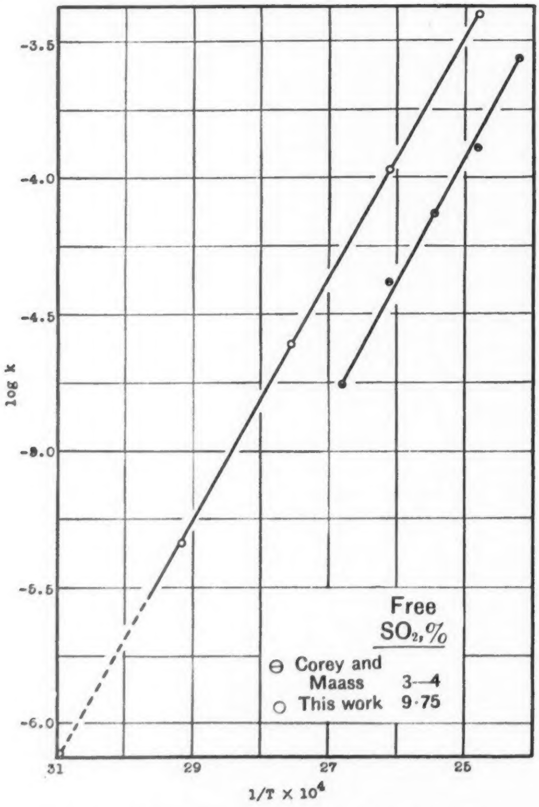

FIG. 4. *The temperature coefficient of delignification.*

is used, an excellent straight line is obtained by plotting the logarithm of velocity against $1/T$, as seen in Fig. 4. The data of Corey and Maass (3), converted from hours^{-1} to seconds^{-1} for comparison, are also plotted in Fig. 4. The magnitudes of the velocities in the two cases differ because different liquor concentrations were used, but the slopes of the two lines (and hence the values of E) are almost identical.

The constants of the Arrhenius equation for the delignification of wood were calculated for our data from the straight line in Fig. 4 and found to be:

$$E = 20{,}200 \text{ cal. per gm. mole}$$

$$Z = 3.42 \times 10^7 \text{ sec.}^{-1}$$

The variation in the rate of delignification with temperature for the sulphite process under the experimental conditions chosen is therefore expressed by the equations:

$$\ln k = 17.3 - 20{,}200/RT \tag{3}$$

or

$$k = 3.42 \times 10^7 \, e^{-20{,}200/RT} \, . \tag{4}$$

The value of E reported by Corey and Maass is 21,000 cal. per gm. mole, which is good agreement when the errors involved are considered. The constant Z, of course, depends on the concentration of the cooking liquor.

The fact that the delignification of wood in sulphite liquor obeys the Arrhenius equation over the temperature range investigated shows that there is no critical temperature, such as 110° C., below which the reaction does not proceed normally. The temperature appears to affect only the velocity of delignification as in the case of comparatively simple chemical reactions. This is true even if we consider the velocity constant calculated at 50° C. unreliable for the reasons already stated. The cooks made at 70° and 90° C. provide sufficient evidence that the reaction obeys the Arrhenius equation below 110° C.

Discussion

The Temperature Coefficient of Delignification

The fact that the delignification reaction obeys the Arrhenius equation rigidly over a considerable temperature range, and in spite of all the complex factors involved, suggests the possibility of a conventional interpretation based on the activation theory of molecular collisions. The authors have shown previously (1) that the active cooking agent in calcium-base sulphite liquor appears to be free sulphurous acid or the hydrogen and bisulphite ions together, and that cooking action cannot be attributed to either ion alone. Let us suppose then, that delignification consists essentially of a reaction between lignin and sulphurous acid. Yorston (14) has provided considerable evidence that all parts of the lignin are accessible to the reagent, and the diffusion rates (in the case of wood-meal) appear to be high enough that we may neglect, for the moment, the heterogeneous nature of the reaction.

In the case of the delignification of wood, we do not know what the lignin "molecule" is. Nevertheless, lignin may be pictured as existing in a number of repeating units or aggregates of unknown size, which in the presence of a peptizing medium may undergo slight molecular motion within the confines allowed by the structure as a whole. The combination of a lignin unit with one or more molecules of sulphurous acid yields a lignin sulphonic acid which is subsequently dissolved or peptized away. The fact that the reaction involves two molecular species, but is not second order, may be explained by the large excess of sulphurous acid. That is, the reaction is merely pseudo-unimolecular. This would indicate a linear relation between the rate of delignification and the concentration of free sulphur dioxide which is approximately borne out by experiment (1).

Moelwyn-Hughes (8) has derived a theoretical value of Z for all uncomplicated bimolecular reactions in solution which is of the order of 10^{11} sec.$^{-1}$ This is due to the compensating effect of two opposing factors, *viz.*, the larger the molecule, the smaller is its velocity, but the larger is its collision area. Consequently, the theoretical collision frequency is approximately constant, and the theoretical reaction velocity will be determined by the energy required for activation. In the delignification of wood, the experimental value of Z is of the order of 10^7 sec $^{-1}$. In other words, the collision frequency appears to be lower than the theoretical value by a factor of 10^4. Or rather, only one collision in 10,000 having the requisite energy (20,200 cal. per gm. mole) results in reaction. This abnormally low velocity has been observed with a number of relatively simple reactions in solution known to be bimolecular, for which Moelwyn-Hughes offers a number of explanations. The most probable one in the present case is that stringent conditions of orientation are required. In view of the size and complexity of the lignin molecule, it is not unreasonable that a reaction should take place only when an activating collision occurs at a certain point.

The value of the critical energy E found for the sulphite process (20,200 cal. per gm. mole) is of the same order of magnitude as that for homogeneous bimolecular reactions in solution. This value may be compared with 32,000 cal. reported by Larocque (7) for the alkali process.

The Rate of Delignification at a Fixed Liquor Composition

Since there is always a large excess of sulphurous acid present during the delignification reaction, we would expect the instantaneous rate of removal of lignin in any one sulphite liquor to be proportional to the quantity of lignin remaining undissolved (*i.e.*, a first order reaction) provided:

(a) That all the lignin is equally accessible to the reagent,
(b) That the lignin is chemically homogeneous,
(c) That only one chemical reaction takes place, or, if there is a series of consecutive reactions, one reaction is slow enough in comparison with the others to be rate determining.

Experimentally, the delignification reaction closely approximates, but does not actually follow, the monomolecular law. The regular deviations from the first order relation cannot be due to the consumption of sulphur dioxide as the reaction proceeds, since the shape of the delignification curve was found to be the same over a wide range for all the liquor concentrations tried (1). In addition, the analysis of the liquor after cooking showed that only very slight concentration changes occurred during cooking. The deviation is therefore real.

The evidence of Yorston (14) is fairly strong that all the lignin is accessible to the reagent. Hence, the consistent departure of the reaction from the first order relation may be due to the non-fulfilment of conditions *(b)* or *(c)*. Since lignin is a complex biological product, there is reason to believe that it is not chemically homogeneous. Lignin sulphonic acids recovered from waste sulphite liquor can be separated into two distinct fractions, although it has been suggested that the difference is one of molecular aggregation rather than chemical behaviour. In addition, the decrease in rate toward the end of the reaction has frequently been attributed to the existence of a more resistant fraction of lignin.

Yorston (14) has developed the hypothesis of two consecutive monomolecular reactions, and has shown that the shape of the delignification curve can be explained on this basis, provided that the velocity constants of the two reactions do not differ by a factor of more than about 2. It was suggested that the rate of sulphonation might be proportional to the quantity of unsulphonated lignin present at any instant, and that the rate of removal of lignin might be proportional to the quantity of sulphonated lignin present at any instant. However, it was found that the actual rate of removal of lignin could not be predicted in this manner, and it was concluded that more sulphur than that required for delignification was taken up by the lignin.

The actual mechanism by which sulphonated lignin is removed from the wood has still to be explained. The delignification mechanism proposed by Hägglund (4, 5) is based on the assumption of a rapid sulphonation reaction, followed by the hydrolysis of a hypothetical lignin-sulphonic-carbohydrate complex, the rate of which is supposedly governed by the hydrogen ion concentration of the liquor which would determine the over-all rate of cooking. This theory is quite untenable since the authors have shown (1) that the rate of delignification is not controlled by the hydrogen ion concentration alone, even in the final stages of the reaction when the lignin is highly sulphonated.

Kullgren (6) has proposed a slightly different mechanism, suggesting that there is an equilibrium between the free lignin sulphonic acid and its calcium salt, and that only the former is soluble. He maintains that the rate of delignification is determined by the ratio of the hydrogen ion concentration to the calcium ion concentration, *i.e.*, (H)/(Ca). The authors' results show that this theory is equally untenable, since the rate of delignification was found to be governed by the product of the concentrations of the hydrogen and bisulphite ions. When the concentration of base is kept constant, the rate of reaction does increase with increase in the hydrogen ion concentration, as Kullgren states. However, an increase in the concentration of base also increases the rate of delignification, provided that the hydrogen ion concentration is held constant. This is exactly the reverse of what Kullgren's theory would predict, and is due to the fact that he has ignored the important role played by the bisulphite ion.

The authors' results indicate that whether there is a hydrolysis reaction or not, the sulphonation reaction is more probably the important rate-determining reaction. Since solubility depends on molecular size as well as on chemical similarity, there are two ways in which the lignin may be rendered soluble which do not involve a hypothetical hydrolysis reaction. The introduction of sulphonic acid groups may confer hydrophilic properties on lignin, allowing it to be more easily peptized in a medium of suitable ionic concentration. A depolymerization or rupture of bonds between lignin units may also occur. Both of these processes would tend to increase the solubility of lignin. The greater the depolymerization or the greater the degree of sulphonation, the more readily would the lignin be peptized. Neither process alone seems to be sufficient. Whether there could be any interrelation between the sulphonation reaction and a depolymerization process cannot be stated at the present time. These points cannot be cleared up until more is known concerning the structure and chemistry of lignin itself.

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A STUDY OF THE COLORIMETRIC DETERMINATION OF PYRROLE WITH ISATIN AND THE APPLICATION OF THE METHOD TO BIOLOGICAL MATERIALS[1]

By G. H. Guest[2] and W. D. McFarlane[3]

Abstract

Fromm's method for the determination of minute amounts of pyrrole has been modified and applied to the determination of pyrrole produced by the dry distillation of various substances. The pyrrole obtained from gelatin is derived entirely from the proline and hydroxyproline which it contains. Enzymatic, acid, or alkali hydrolyzates of gelatin, gliadin, and glutenin, contain no detectable amount of free pyrrole. Sodium peroxide markedly increases the yield of pyrrole obtained in the dry distillation of gelatin. The addition of copper sulphate catalyzes the oxidation of proline to pyrrole by sodium peroxide.

Introduction

Two colorimetric reactions have been widely employed for the determination of minute amounts of pyrrole, namely, the production of a pink colour with *p*-dimethylaminobenzaldehyde and the formation of pyrrole-blue by condensing pyrrole with isatin in acid solution. The former reaction is lacking in specificity, being given by substituted pyrroles, pyrrolines, pyrrolidines, and indoles. On the other hand, the isatin reaction is highly specific, being given only by pyrroles with an α-position unsubstituted, and not given by reduced pyrroles or indole derivatives.

Fromm (2) has recently developed for the determination of pyrrole a colorimetric method that employs the isatin reaction. The authors have had occasion to use this method and have found it necessary to make several modifications, which are described in this paper. The application of the method to the determination of pyrrole in biological materials requires the separation of the pyrrole from other decomposition products which interfere with the reaction. For this purpose the conditions for the quantitative precipitation of minute amounts of pyrrole by mercuric chloride, and for the complete extraction of pyrrole by ether, have been investigated. The procedure developed has been applied to the determination of the yield of pyrrole obtained by the dry distillation of various proteins and other substances, and also to determine whether free pyrrole occurs in protein hydrolyzates.

Experimental

I. An Improved Micro-colorimetric Method for the Determination of Pyrrole as Pyrrole-blue

To determine the optimum conditions for the development of the blue colour in the pyrrole-isatin reaction, experiments were conducted with a

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solution of freshly distilled pyrrole in 0.5% acetic acid. This solution is stable for at least one month, whereas a solution of pyrrole in 0.5% hydrochloric acid develops a yellow colour, with appreciable loss of pyrrole, within 24 hr. Fromm (2) carried out the procedure by adding 2 ml. of 6 *N* hydrochloric acid to an aliquot of a solution of pyrrole in 0.5% hydrochloric acid and diluting to 10 ml. To this solution was added 1 ml. of a 0.05% solution of isatin in glacial acetic acid and the mixture heated for 10 min. in a boiling water bath. After the solution had been cooled, the intensity of the blue colour was measured with a step-photometer.

The following observations of Fromm's procedure were made with the aid of a single-cell type of photoelectric colorimeter with light filters, as described by Evelyn (1):— *(a)* Increasing the concentration of hydrochloric acid markedly increased the intensity of the blue colour, the maximum being obtained when the reaction was carried out in 5 to 6 *N* hydrochloric acid. With this concentration of hydrochloric acid the maximum intensity of colour was developed on standing for five minutes at room temperatures. By eliminating the heating on the boiling water bath more reproducible results were obtained. *(b)* It was found that 0.2 ml. of an 0.5% solution of isatin in glacial acetic acid gave the most satisfactory results. More of this reagent slightly increased the intensity of the blue colour but gave too high a galvanometer reading in the control determination. *(c)* Dilution of the reaction mixture with 95%

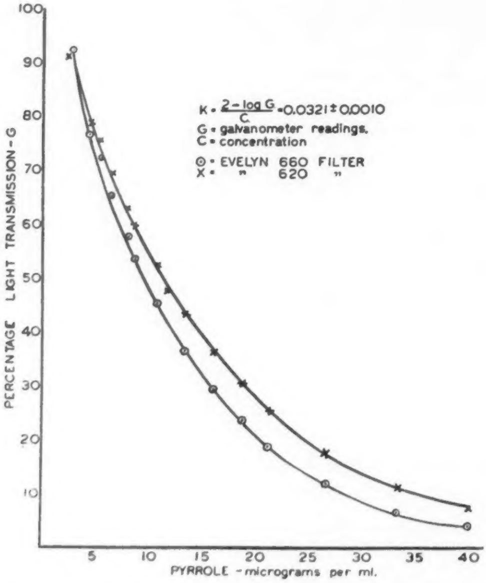

FIG. 1. *Showing the relation between the concentration of pyrrole and the intensity of the pyrrole-blue colour.*

ethyl alcohol instead of water gave a much more intense and more stable blue colour.

The procedure finally adopted was as follows:— 1 ml. of the pyrrole solution, containing 5-30 micrograms of pyrrole per ml., was transferred to a 10 ml. glass-stoppered graduated cylinder and 0.2 ml. of a 0.05% solution of isatin in glacial acetic acid and 1 ml. of concentrated hydrochloric acid were added. After five minutes the mixture was diluted to the 10 ml. mark with 95% ethyl alcohol and the intensity of the blue colour measured with an Evelyn photoelectric colorimeter, using a 660 mu filter. The centre setting of the instrument was determined with a blank solution, prepared in a manner identical with that of the test solution but to which no pyrrole was added. The colour was stable for at least one hour and no significant difference in the colour intensity was observed if the reaction time was varied from 5 to 20 min. The calibration data are shown in Fig. 1 and were obtained by the application of the above procedures to a standard solution of pyrrole. Under these conditions the isatin-pyrrole reaction conforms to Beer's law, the mean value of K being 0.0321 ± 0.0010. The calibration data obtained with the two filters were used to detect any change in the shade of colour particularly when the method was applied to crude materials. The authors have observed that 2,5-dimethylpyrrole, 2-carbethoxypyrrole, 1,2-dicarbethoxypyrrolidine, pyrrolidine, proline, hydroxyproline, tryptophane, and indole failed to react with isatin under the conditions of the test, but 1-carbethoxypyrrole did react.

Fromm (2) has further reported an investigation of the use of mercuric chloride as a precipitant to separate pyrrole from substances which would interfere with the colour reaction. The authors have repeated his experiments and have been unable to confirm the quantitative recovery of pyrrole after precipitating with mercuric chloride, washing the precipitate with alcohol, and finally dissolving the precipitate in 5% sodium cyanide solution. The precipitate is appreciably soluble in 95%, or absolute, ethyl or methyl alcohol; the pyrrole-blue colour is modified by the presence of sodium cyanide, and, further, quantitative precipitation of pyrrole by mercury depends on the pH of the solution, the best results being obtained by buffering the solution with phosphate buffer of pH 6.3.

The procedure which the authors have finally adopted is as follows:— 5 ml. of pyrrole solution, containing 0.158 mg. of pyrrole per ml., were placed in a 15 ml. graduated centrifuge tube and to it was added 5 ml. of a phosphate buffer solution (pH 6.3), and 5 ml. of a saturated aqueous solution of mercuric chloride. The mixture was vigorously stirred and allowed to stand for one hour before centrifuging. The precipitate was washed twice with 10 ml. portions of distilled water. After each washing the supernatant liquid was removed carefully by suction. The precipitate was dissolved in 15 ml. of 0.5% hydrochloric acid solution and made up to 50 ml. with distilled water. Application of the isatin reaction to a 1 ml. aliquot of this solution showed a recovery of 96.47% of the pyrrole.

Substances which interfere with the colour reaction may also, in some cases, be eliminated by extracting the pyrrole with ether. In order to effect a quantitative recovery, however, certain precautions must be taken which have not heretofore been recognized. The following experiment illustrates the procedure which the authors have employed successfully:— 10 ml. of a pyrrole solution containing 1.51 mg. of pyrrole was extracted by shaking with 10 ml. of ether in a small separatory funnel. The extraction was repeated five times and the extracts were combined. The ethereal solution was dropped slowly into a suction flask containing 10 ml. of 5% acetic acid solution which was placed in a beaker of warm water (50 to 60° C.) and connected to the suction pump through a trap which also contained acetic acid solution. When all the ether had evaporated, the acetic acid solutions in the trap and in the suction flask were combined and made up to 100 ml. with water. Colorimetric determination of the pyrrole contained in a 1 ml. aliquot of this solution showed 94% recovery, whereas in the absence of the trap the recovery was only 27%.

The methods described above have been successfully employed in the determination of pyrrole in the decomposition products of biological materials.

II. The Determination of Pyrrole in the Decomposition Products of Biological Materials

It has long been known that many biological materials, particularly proteinaceous substances, yield pyrrole by dry distillation. The amount of pyrrole obtainable by the dry distillation of various substances is shown in Table I. These determinations were carried out as follows:— A weighed sample, usually about 0.1 gm. of the dry, powdered substance was introduced into a soft-glass test tube ($\frac{5}{8}$ × 6 in.), and the centre of the tube, after being softened in a flame, was drawn out to a construction of about 5 mm. and bent at an angle of about 45°. The tube was held in a clamp so that its mouth was below the surface of 10 ml. of a 5% solution of acetic acid contained in a

TABLE I

SHOWING THE YIELD OF PYRROLE OBTAINED BY THE DRY DISTILLATION OF VARIOUS SUBSTANCES

Substance	Yield of pyrrole, %	Substance	Yield of pyrrole, %
Gelatin	1.201 ± 0.075*	Ammonium mucate	37.15
Casein	0.14	Proline	1.83
Gluten	0.18	Hydroxyproline	7.31
Gliadin	0.22	Arginine	Trace
Glutenin	0.16	All other amino acids found in proteins, and ammonium salts of these amino acids	Nil
Ovalbumin	0.13		
Lactalbumin	0.03		
Edestin	0.06		
Chlorophyll	Nil		

* *Nine determinations.*

30 ml. beaker. The material was heated, gently at first, and finally to redness by applying a micro flame to the rounded base of the test tube. Heating was continued until no more vapour distilled over. The delivery tube was severed just above the shoulder and used as a funnel to transfer the contents of the beaker to a 100 ml. volumetric flask. This was found to be the simplest and most efficient of the many types of distillation apparatus tried. The isatin colorimetric determination was made, in the usual manner, a 1 ml. aliquot of the final solution being employed. It was found necessary, however, to remove coloured impurities from the distillate. The solution could be decolourized without loss of pyrrole, with Lloyd's reagent, about 0.2 gm. being employed to remove the colour from 5 ml. of the distillate.

It was noted that the yield of pyrrole obtained by the dry distillation of various proteins (Table I) amounted to what might be expected from their proline and hydroxyproline content if, as was found to be the case, only these two amino acids yield pyrrole on dry distillation. From their proline and hydroxyproline content, and the yield of pyrrole obtained by the dry distillation of these amino acids, the amount of pyrrole obtained from a number of proteins can be accounted for (see Table II). This observation is substantiated by the finding that when a hydrochloric acid hydrolyzate of 500 gm. of gelatin was fractionated by the classical methods the pyrrologenic material was found in the proline-hydroxyproline fraction. This material showed the following properties:— it was not precipitated by basic lead acetate nor by phosphotungstic acid; it was soluble in hot absolute alcohol; and 12% of it was precipitated in alcoholic solution by cadmium chloride (proline fraction). The alcohol soluble material gave a negative reaction to Foulger's modification of Molisch's test (3).

TABLE II

SHOWING THAT THE YIELD OF PYRROLE OBTAINED BY THE DRY DISTILLATION OF SEVERAL PROTEINS IS ACCOUNTED FOR BY ASSUMING THAT ONLY THE PROLINE AND HYDROXYPROLINE, WHICH THEY CONTAIN, YIELD PYRROLE

	Pyrrole by distillation, %	Proline (6), %	Hydroxyproline (6), %	Pyrrole by calculation, %
Gelatin	1.20	9.5	14.1	1.20
Casein	0.14	9.0	0.2	0.17
Gliadin	0.22	13.2	—	0.24

Pieroni (4) and Roncato (5) have reported the presence of pyrrole among the hydrolytic products of glutenin and gliadin. Using the sensitive isatin test the authors have failed to detect any free pyrrole in the hydrolyzates when gluten, gliadin, or gelatin were digested with pepsin, trypsin, pepsin followed by trypsin, papain, 10 or 20% sodium hydroxide, 25% hydrochloric acid, or 25% sulphuric acid, nor was any pyrrole detected in the steam distillates of any of these hydrolyzates.

The authors have further examined the effect of several oxidizing agents on the yield of pyrrole obtained by the dry distillation of gelatin. The addition of ferric chloride failed to alter the yield, and no pyrrole was obtained when the dry distillation was carried out after the gelatin had been treated with sodium hypochlorite, hydrogen peroxide, or alkaline permanganate. However, it was found that sodium peroxide did markedly increase the amount of pyrrole obtained from gelatin (see Table III). In Table IV the effect of the addition of copper sulphate is shown. These experiments suggest that the hydroxyproline and proline molecules differ greatly in their susceptibility to oxidation. It would appear that with hydroxyproline the oxidation was too vigorous when copper sulphate and sodium peroxide were added together. Proline, on the other hand, is apparently more difficult to oxidize, since, when the copper sulphate was omitted, the sodium peroxide alone failed to bring about its oxidation to pyrrole.

TABLE III

THE EFFECT OF SODIUM PEROXIDE ON THE PRODUCTION OF PYRROLE FROM A GELATIN HYDROLYZATE

Weight of sodium peroxide added to 80 mg. of gelatin comminuted with 5 gm. of sand, gm.	0.25	0.50	0.50	0.50	1.00
Increase in yield of pyrrole, %	85	180	200	190	105

TABLE IV

THE EFFECT OF COPPER SULPHATE ON THE PRODUCTION OF PYRROLE

Composition of test	Yield of pyrrole, %
100 mg. gelatin + 1 ml. H_2O	0.93
100 mg. gelatin + 1 ml. H_2O + 0.5 gm. Na_2O_2	2.65
100 mg. gelatin + 0.5 gm. Na_2O_2 + 1 ml. 10% $CuSO_4$	2.00
10 mg. proline + 2 ml. H_2O + 0.5 gm. Na_2O_2	Nil
10 mg. proline + 2 ml. H_2O + 0.5 gm. Na_2O_2 + 1 ml. 10% $CuSO_4$	8.44
10 mg. hydroxyproline + 2 ml. H_2O + 0.5 gm. Na_2O_2	7.25
10 mg. hydroxyproline + 2 ml. H_2O + 0.5 gm. Na_2O_2 + 1 ml. 10% $CuSO_4$	3.25

Acknowledgment

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A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF HYDROXYPROLINE AND ITS APPLICATION TO GELATIN HYDROLYZATES[1]

By W. D. McFarlane[2] and G. H. Guest[3]

Abstract

Hydroxyproline is oxidized by sodium peroxide, if copper or cobalt is present, to give an unstable red chromogen which, in hot acid solution, can be condensed with isatin to form a stable red complex. The conditions are described which permit the direct application of this reaction to the quantitative estimation of hydroxyproline in acid or alkali hydrolyzates of gelatin. By this method moisture-free gelatin is found to contain 14.65% of hydroxyproline.

Introduction

An examination of the literature reveals that few colour reactions are available for the detection and determination of hydroxyproline. Morse (5) introduced a new colour reaction, namely, the production of a red colour when a solution of hydroxyproline is heated with sodium peroxide in the presence of methylhexylcarbinol. A quantitative procedure based on this reaction has not so far been evolved, and in the authors' tests the reaction has been found to take place with explosive violence. Lang (4) and Waldschmidt-Leitz and Akabori (6) have shown that hydroxyproline reacts with sodium hypochlorite to yield 80% of pyrrole. The latter can then be determined colorimetrically, with *p*-dimethylaminobenzaldehyde or isatin, in the steam distillate. The hydroxyproline content of gelatin as determined by this procedure is, however, only 8.9 to 9.4% which is very low when compared to the amount obtained by isolation methods, *i.e.*, 14.1% by Dakin (2) and 14.4% by Bergmann (1).

It has been demonstrated (3) that there is a marked difference in the susceptibility of proline and hydroxyproline to oxidation. The authors have also found that, if a solution of hydroxyproline is treated with sodium peroxide and dilute copper sulphate solution and the mixture acidified with hydrochloric acid, a red colour develops on heating the solution in a boiling water bath. This constitutes a sensitive test for hydroxyproline which is not given by any other amino acid, including proline. The reaction does not take place if the copper sulphate is omitted. A method for the determination of hydroxyproline, based on this reaction, is described herein; when applied to gelatin hydrolyzates it gives results in good agreement with the values obtained by isolation methods.

Experimental

The qualitative test for hydroxyproline is carried out as follows:— To 1 ml. of a hydroxyproline solution or gelatin hydrolyzate containing at least 0.5

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mg. of hydroxyproline is added approximately 0.25 gm. of sodium peroxide and 1 ml. of 0.02 *M* copper sulphate solution. When effervescence has ceased, the mixture is neutralized with 2 *N* hydrochloric acid solution, with phenolphthalein as an indicator, and then an excess of hydrochloric acid is added to give a final concentration of approximately 0.1 *N* acid. The solution is placed in a boiling water bath for a few minutes and a red colour develops.

When the copper sulphate is replaced with an equimolecular solution of ferric sulphate, nickelous sulphate, or manganous sulphate no colour develops, but, from a given amount of hydroxyproline, cobaltous sulphate produces about one-third of the colour intensity given by copper sulphate. If hydrogen sulphide is used to remove the copper, just before the solution is finally heated in the water bath, the red colour fails to develop. The chromogen is insoluble in ether; it is not precipitated by mercuric sulphate and it gives no colour with isatin until the mixture is heated, when a red colour develops which, for a given amount of hydroxyproline, is more intense and more stable than the red colour developed on heating with hydrochloric acid alone. The substance may possibly be a complex copper salt of a dipyrryl methene.

A study of this reaction to determine its value as the basis of a quantitative procedure showed:—

(a) The intensity and the shade of the red colour are influenced by factors which are difficult to control. If, however, the chromogen is condensed with isatin the red colour which now develops is stable, and the conditions under which the colour intensity is proportional to the concentration of hydroxyproline can be established.

(b) The absorption curve of the red colour shows a maximum at about 520 mu, so that the intensity can best be measured in the Evelyn photoelectric colorimeter by employing the 520 mu filter.

The absorption curve of the red colour, obtained by applying the reaction to a gelatin hydrolyzate, is identical with that of the red colour developed from hydroxyproline.

(c) The amount of copper sulphate influences the reaction. Maximum colour intensity is obtained, if the reaction is carried out, with a 0.01 *M* copper sulphate solution.

(d) The use of solid sodium peroxide gives variable results. This difficulty is overcome by using instead 1 ml. of 6% hydrogen peroxide and 1 ml. of 10% sodium hydroxide solution.

The effect of *(a)* the temperature and duration of the heating of the reaction mixture, *(b)* the concentration of hydrochloric acid and isatin, and *(c)* the temperature and time of heating on the development of the red colour, have been investigated. The optimum conditions are given in the following description of the method.

Description of the Method

To 1 ml. of a solution containing 0.2 to 1.6 mg. of hydroxyproline are added 1 ml. of 0.01 *M* copper sulphate solution, 1 ml. of 10% sodium hydroxide solution, and 1 ml. of 6% hydrogen peroxide. The mixture is allowed to stand for five minutes, with shaking at frequent intervals. The test tube is then placed in a boiling water bath for five minutes, cooled in running water, and neutralized by the addition of 1.5 ml. of 2 *N* hydrochloric acid solution. The solution is next transferred to a 10 ml. glass stoppered graduated cylinder and diluted to the 10 ml. mark with distilled water. After thorough mixing, 1 ml. of this solution is transferred to another 10 ml. graduated, glass stoppered cylinder; 1 ml. of a freshly prepared 0.01% aqueous solution of isatin and 1 ml. of 2 *N* hydrochloric acid solution are added, and the mixture is placed in a boiling water bath for exactly three minutes. The cylinder is removed from the water bath, allowed to stand for five minutes, and then cooled under running water. Water is then added to the 10 ml. mark, and, after mixing, the red coloured solution is transferred to a colorimeter tube. The intensity of the colour is measured in the photoelectric colorimeter, using the 520 light filter.

The heating times mentioned above should be closely adhered to. The isatin solution should be not more than two days old, and in its preparation gentle warming may be employed to facilitate solution of the isatin. The centre-setting of the colorimeter was obtained by using a control solution prepared by the same procedures, but with the hydroxyproline omitted. In Fig. 1 are shown the calibration data obtained with solutions containing varying amounts of hydroxyproline. The reaction conforms with Beer's law, the value of K being 0.3483 ± 0.0095 with a maximum deviation of 0.0166.

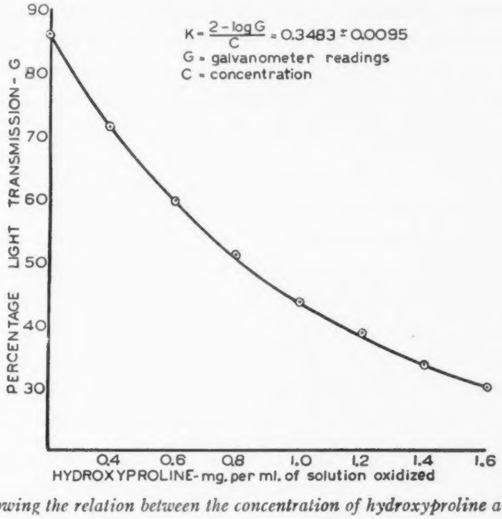

FIG. 1. *Showing the relation between the concentration of hydroxyproline and the intensity of the red colour.*

Application of the Method to Gelatin Hydrolyzates

Various methods of hydrolysis have been tested, with the results shown in Table I. In each case 0.5 gm. of finely powdered "Gold Label" gelatin, which analyzed 10.17% moisture and 17.46% nitrogen (moisture-free basis), was employed.

TABLE I

SHOWING THE RESULTS OF HYDROXYPROLINE DETERMINATIONS IN GELATIN HYDROLYZATES

Method of hydrolysis	Hydroxyproline, % of moisture-free gelatin
3 *N* HCl at 150° C. for three hours	14.19
3 *N* HCl at 150° C. for five hours	14.34
3 *N* HCl at 150° C. for six hours	14.53
3 *N* HCl at 150° C. for six hours	14.67
Saturated $Ba(OH)_2$ at 150° C. for three hours	13.07
Saturated $Ba(OH)_2$ at 120° C. for six hours	14.75

Hydrolysis was carried out in an autoclave at 150° C. and a pressure of of 70 lb., using 10 ml. of 3 *N* hydrochloric acid or 20 ml. of a saturated solution of barium hydroxide. At the completion of the acid hydrolysis the solutions were neutralized to a phenolphthalein end-point with sodium hydroxide, whereas the alkali hydrolyzates were neutralized by adding 5 ml. of 10% sulphuric acid to the hot solution. Each hydrolyzate was filtered into a 50 ml. volumetric flask and the precipitate on the filter paper washed with water until the total volume of the filtrate was 50 ml. The colorimetric determinations were carried out in duplicate, using 1 ml. aliquots of this solution. The "centre setting" of the colorimeter was obtained with a control solution which was prepared by treating 1 ml. of the hydrolyzate in the same way as the test solutions were treated, with the exception that it was not heated after addition of the isatin and hydrochloric acid solutions. This solution did not therefore develop a red colour, and it was used to compensate for the colour of the reagents.

The three lowest results in Table I probably indicate incomplete hydrolysis, if these are omitted the mean value for the hydroxyproline content of gelatin becomes 14.65% (moisture-free basis). So far, the authors have been unable to detect hydroxyproline in casein, although the amount of the sample taken for analysis was such that hydroxyproline should have been detected if casein contains 0.2%.

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A NOTE ON THE COLORIMETRIC DETERMINATION OF PROLINE[1]

By G. H. Guest[2]

Abstract

A colorimetric method for the determination of proline is described. The procedure involves the oxidation of proline with lead peroxide and the condensation of the oxidation product with *p*-dimethylaminobenzaldehyde to give a red compound. The oxidation product can, if desired, be separated by steam distillation before applying the colour reaction. The method is limited in application to proteins which contain little or no hydroxyproline. By this method casein is found to contain 7.94% of proline.

Introduction

When a mixture of proline with sodium peroxide and copper sulphate is dry distilled, pyrrole is present in the distillate as indicated by a positive pyrrole-blue test with isatin (3). Proline or hydroxyproline, on oxidation with sodium peroxide in the presence of copper sulphate, gives oxidation products which react, in acid solution, with *p*-dimethylaminobenzaldehyde. The oxidation product of hydroxyproline, but not of proline, gives a red colour on condensing with isatin in acid solution (4). Determination of the relative intensity of the red colour produced, in the reaction with *p*-dimethylaminobenzaldehyde, from the same amount of proline and hydroxyproline under identical conditions, shows that 1 mg. of hydroxyproline produces the same intensity of colour as 10 mg. of proline. These observations suggested the possibility of evolving a method for determining proline and hydroxyproline in protein hydrolyzates. Such a procedure was worked out, but when it was applied to gelatin hydrolyzates the results were always much lower than those recorded in the literature and obtained by isolation methods (1).

It was considered that it would be necessary to separate the compound which produced the red colour with *p*-dimethylaminobenzaldehyde from interfering substances. The oxidation product of proline or hydroxyproline cannot be steam distilled nor can it be extracted with ether or precipitated with mercuric salts. It has been found, however, that lead dioxide will oxidize proline and hydroxyproline to products which give a red colour with *p*-dimethylaminobenzaldehyde and which are also volatile in steam.

After a careful study of the optimum conditions for the oxidation of proline by lead dioxide and for the colorimetric determination with *p*-dimethylaminobenzaldehyde, a procedure has been evolved for the determination of proline. This method has, so far, been found to be applicable only to proteins, such as caseinogen, which contain little or no hydroxyproline.

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Description of the Method

To 2 gm. of casein in a 50 ml. Erlenmeyer flask was added 40 ml. of a saturated solution of barium hydroxide. The flask was placed in an autoclave for three hours at 150° C. and a pressure of 70 lb. On removal of the flask from the autoclave 10 ml. of 10% sulphuric acid solution was added to the contents of the flask and, after cooling, the mixture was filtered into a 100 ml. volumetric flask. The residue was washed on the filter paper, with distilled water until 100 ml. of filtrate was collected.

A 5 ml. aliquot of the filtrate was neutralized to phenolphthalein with 1 *N* sodium hydroxide solution, and to it were added 5 ml. of water, 10 ml. of phosphate buffer (pH 8.7) and 1 gm. of lead dioxide. The mixture was refluxed for 30 min., cooled, filtered into a 200 ml. volumetric flask, the residue washed with water, and the filtrate finally made up to volume. To 5 ml. of this solution in a glass-stoppered graduated cylinder were added 5 ml. of water, 1 ml. of a 4% solution of *p*-dimethylaminobenzaldehyde in 95% alcohol, and 1 ml. of 2 *N* hydrochloric acid. The mixture was heated in a boiling water bath for one minute, and cooled by allowing to stand for five minutes and then placing in cold running water. The colour intensity was measured in an Evelyn photoelectric colorimeter using the 520 mu light filter.

The proline equivalent was calculated by referring to a calibration curve which was prepared from the data obtained by applying the above reactions to amounts of proline varying from 1 to 5 mg. The colour reaction conforms with Beer's law, the mean value of K being 0.0578 ± 0.0020 with a maximum deviation of 0.0051.

This sample of casein was found to contain 7.94% proline (moisture-free basis) which is in close agreement with the value obtained by Dakin (2) by his butyl alcohol method.

It will be noted that, in the application of the method to casein, steam distillation was not employed. A slightly lower value for the proline content of casein is obtained if steam distillation is introduced, but in this particular case no advantage was found to accrue from its use. Smaller amounts of the protein may, of course, be employed.

Possibly the method can be modified to be applicable to proteins containing hydroxyproline so that hydroxyproline and proline may be simultaneously determined in the same hydrolyzate. However, circumstances have made it necessary that this investigation be discontinued for the present.

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NOTE ON PERSISTENCE OF LIQUID STRUCTURE IN THE CRITICAL REGION

Investigations carried out in this laboratory have shown that above the critical temperature, as defined by the temperature of the disappearance of the meniscus, a heterogeneity in a one component system persisted. By "molecular stirring", by heating the medium considerably above this temperature and then reproducing the original temperature, or by isothermal expansion, this heterogeneity disappeared and a homogeneous system was obtained which persisted indefinitely. Up till now it has been impossible to produce a heterogeneous system once the critical temperature, as defined above, has been passed. It is of considerable importance to record that this is possible.

The two phase system ethylene was heated to above the critical temperature and the expected heterogeneity recorded. It was then heated up five degrees above the critical and on cooling to the previously recorded temperature the system was homogeneous. By means of a heating jacket covering the upper portion of the bomb, the lower portion of the medium was subjected to pressure which was maintained for some time. The experimental arrangement was such that the whole system was immersed in a thermostat so that the lower portion of the medium remained at constant temperature. When the temperature of the jacket was allowed to fall to the thermostat temperature a heterogeneity persisted, i.e., the density of the lower portion of the medium reached an asymptotic value definitely higher than that of the upper portion. Such experiments have been found to give reproducible results, and it has therefore been shown that above the critical temperature it is possible to produce in a one component system a dense and a less dense medium apparently in equilibrium with each other. The inferences to be drawn are left to the detailed publication of the results of these experiments.

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